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Anionic Dependence of Secretion and Secretory Potentials in the Perfused Sublingual Gland.

By

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On the basis of electrophysiological experiments the hypothesis was proposed that the secretory potential over the outer membrane of sublingual gland cells is due to an active transport of chloride and that secretion of salt and water is a consequence of this transport (LUNDBERG 1957 b).

The present experiments were planned as a further test of this hypothesis of secretion. It will be shown that the perfused sublingual gland functions normally in the absence of any other anion in the external medium than chloride, whereas with only nitrate ions in the external fluid the secretory potential over the outer membrane decreases considerably and the amount of secretion drops to 10—25 per cent. A preliminary report of this work has been published (LUNDBERG 1956).

Methods.

For perfusion of the gland the attempt was made, whenever possible to ligate all other branches from the external maxillary artery except those to the sublingual gland, but sometimes, when it was difficult to ligate all branches to the submaxillary gland, this gland was perfused as well. For perfusion a polyethylene tube was introduced into the external maxillary artery distal to the branch for the two glands and the artery was tied centrally to this branch.

A hydrostatic non-pulsatile pressure of 150—200 mm Hg was used and after perfusion had been established satisfactorily, as judged from the outflow in the cannulated vein, the cat was killed in order to avoid admixture of blood from anastomosing vessels in the duct walls. The perfused gland was activated by injections of 1 μg acetylcholine

in 0.1 ml saline. Activation with acetylcholine was a necessity because when perfusing with nitrate-saline the gland could not be made to secrete on stimulation of the chorda, presumably due to a block of synaptic transmission in the parasympathetic ganglion cells. This block was not reversible on return to normal saline. The perfusion fluid had the following cationic composition expressed as m mol/l: Na 143; K 4.3; Ca 2.1. In order to minimize oedema during perfusion 6 per cent macrodex was added to the perfusion fluid, which was passed through glass filters and oxygenated for 60 minutes before it was used. In order to increase the amount of dissolved oxygen and to lower oxygen consumption of the gland, perfusion was carried out at 20° C. For intracellular recording from the perfused gland the microelectrodes were filled with 3 M KNO_3. In order to make inspection of the tubules easier excessive extracellular fluid was continually sucked away through a glass capillary handled by a micromanipulator. For technique of recording see LUNDBERG (1955, 1957 a, b). For measurement of the amount of saliva secreted, the cannula in the duct was connected to a 0.1 ml pipette placed horizontally on level with the gland. Readings could be made with an accuracy of 0.001 ml.

Results.

In order to ascertain that the perfused gland functioned satisfactorily the amounts of saliva on stimulation of the chorda were compared in the blood perfused gland before and after the start of perfusion with saline and were found to be very similar. Furthermore the intracellularly recorded secretory potential resulting from chorda stimulation had approximately the same magnitude and time-course. On injection of 1 μg acetylcholine into the perfusion fluid the outer membrane was found hyperpolarize by up to 42 mV.

In all experiments in which chloride was substituted by other anions activation was made by injection of 1 μg acetylcholine. In glands perfused with chloride-saline the amount of saliva secreted during 3 minutes after such an injection was usually 0.04 ml but varied in different between 0.02—0.06 ml. This is comparable with the amount secreted on stimulation of the chorda for 10—30 seconds. Of the total amount secreted during 3 minutes after the injection of acetylcholine 60—85 per cent was obtained during the first minute after the injection.

EMMELIN (1953) found that there is a continuous spontaneous secretion from the sublingual gland. By experiments on perfused glands and isolated glands in Tyrode solution he showed that the continuous flow is not a filtration through the gland, but repre-

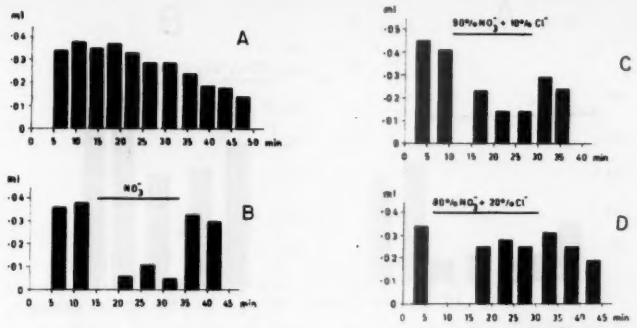

Fig. 1. Secretion in perfused glands. Each black column represents the amount of saliva obtained during 3 minutes after injection of 1 μg acetylcholine. Plot A shows the decline of secretion in gland perfused with saline containing 100 per cent chloride. The other plots (B—D) illustrate experiments in which the glands during time indicated by the black line were perfused with a saline in which chloride was completely or partially replaced by nitrate.

sents a secretory activity. He found an average rate of resting secretion flow in glands with normal blood supply of 0.027 ml per hour. The occurrence of this flow has been confirmed in the present experiments and was of the order of 0.001 ml in 3 minutes, or about 2.5 per cent of the amount secreted during this time by the chloride-perfused gland after injection of acetylcholine. In each experiment the flow of spontaneous secretion was measured before the start of the perfusion and glands with a resting flow larger than 0.001 ml in 3 minutes were not used.

Plot A in Fig. 1 shows an experiment on a gland perfused for 50 minutes with normal chloride-saline. Each black column shows the amount of saliva secreted during 3 min. after injection of 1 μg acetylcholine, and the decline in the response with time after the start of the perfusion is evident. This decline could not be compensated by increasing the dose of acetylcholine and is therefore caused by a decline of the secretory power of the gland, which is probably due to asphyxiation. Sometimes the decrease in function was more rapid and sometimes less than in this case. In one experiment there were less than 20 per cent decline of secretion after two hours' perfusion.

In the experiment illustrated by B in Fig. 1 perfusion was started with chloride-saline, but after 15 min. this saline was exchanged for one containing nitrate instead of chloride as the

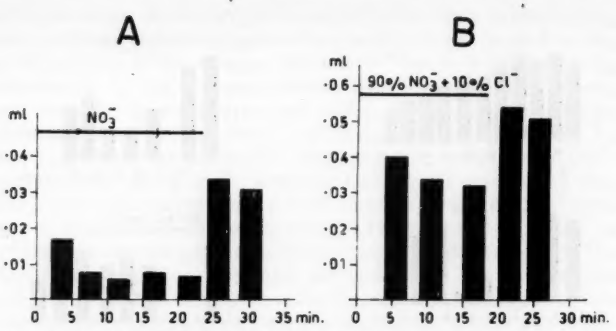

Fig. 2. Secretion in nitrate-perfused gland without an initial period of perfusion with chloride-saline.

only anion. After 5 minutes the amount of saliva in response to acetylcholine had dropped to 15 per cent. Perfusion with nitrate-saline was continued during 18 minutes (during the black line marked NO_3), where after perfusion with chloride-saline was re-established. As seen in the figure there is an immediate return to normal level of secretion when allowance is made for the expected decline in the secretory ability of the perfused gland.

Since the re-establishment of perfusion with chloride causes an immediate recovery from the depression it may be assumed that nitrate ions are not toxic to the secretory mechanism. The depression of secretion by nitrate perfusion was further studied in glands perfused with saline in which chloride was partially substituted by nitrate. In Fig. 1 C can be seen that already with 10 per cent chloride the secretory response is noticeably larger than without chloride and with 20 per cent in D the amount of saliva in response to acetylcholine is not very much smaller than the subsequent amount with 100 per cent chloride.

The results of these perfusion experiments are summarized in Fig. 3. Parts of the experiments included in this graph were made without an initial period of perfusion with full chloride-saline (Fig. 2), and the effect of complete and partial substitution of chloride by nitrate was judged only by the return of the secretory ability after switching to a perfusion fluid containing full chloride. This was done for two reasons: i) Even with macrodex in the perfusion fluid some oedema is unavoidable and the presence of an oedema may delay diffusion equilibrium. ii) Since the perfused gland is deteriorating relatively fast it is desirable to

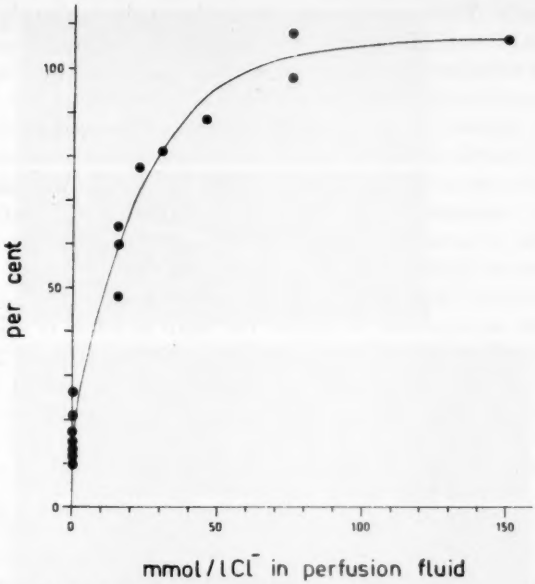

Fig. 3. Graph showing the amount of secretion in response to 1 μg acetylcholine with complete or partial replacement of chloride by nitrate in perfusion fluid. Ordinates, amount of secretion as percentage of that obtained in the same gland after change to a perfusion fluid containing 150 m mol/l chloride.

examine the effect of the changed ionic milieu as soon as possible after the start of the perfusion. The results from the experiments carried out in this way did not differ significantly from those made as described above and they are therefore plotted together in the graph in Fig. 3. In this graph the ordinate represents the amount of secretion in per cent of that obtained in she same gland after change to a perfusion fluid containing 150 mM chloride. Without chloride in the perfusion fluid, secretion is about 10—25 per cent of normal, it increases rapidly with increasing concentration of chloride so that there is virtually no depression of secretion when the chloride concentration is 50 per cent of the normal level. It should be noted that in this graph the curve is drawn to a level somewhat above 100 per cent, which is understandable since the amounts of secretion are measured in per cent of the amount of saliva resulting on a succeeding injection of acetylcholine, and as shown above secretion declines

continually. These experiments give further evidence that the mere presence of nitrate ions is not the cause of the failure of secretion, but that withdrawal of chloride ions is the reason. The limiting factor for secretion at low chloride concentration is presumably depletion of chloride in the external medium of the gland cells. At normal chloride levels the amount of saliva secreted in response to 1 μg acetylcholine is about one fourth of the weight of the gland.

These experiments are in complete accord with predictions from the hypothesis that secretion largely depends on active transport of chloride ions (LUNDBERG 1957 b).

If secretion thus depends on the active transport of chloride ions, how is it possible to explain the small secretion of saliva on injection of acetylcholine in the nitrate-perfused gland? At the most 10—25 per cent of this secretion may be accounted for by the spontaneous secretion which may continue during the whole experiment and which was not subtracted from any of the measurements. It is most unlikely that any residual secretion can be due to chloride ions which remain in the external fluid of the gland cells. With the exception to the first injection of acetylcholine succeeding injections give rather similar amounts of saliva (Figs. 1, 2) which would not be expected if secretion were due to remaining chloride ions. Furthermore, if secretion in the nitrate-perfused gland were only dependent on chloride, secretion in itself would add very markedly to the depletion of chloride in the external medium. In the experiment illustrated in Fig. 2 A the total amount of saliva obtained during perfusion with nitrate roughly corresponds to the total volume of the extracellular space in the gland with normal blood flow. Probably diffusion equilibrium between the perfusion fluid and the extracellular fluid for all practical purposes is reached after about 5 minutes' perfusion.

In order to gain some knowledge about the mechanism of secretion remaining in the nitrate-perfused gland electrophysiological experiments were made on the perfused gland. These experiments are difficult to perform because only a few measurements can be made on each gland before it deteriorates; and, since the units quite often are lost, it is difficult to get reliable records. The conclusions drawn from these experiments are partly based on observations in which the potential change was not observed throughout its full time-course.

The records in Fig. 4 are representative of the potential changes found in the seven experiments that were made. The two upper

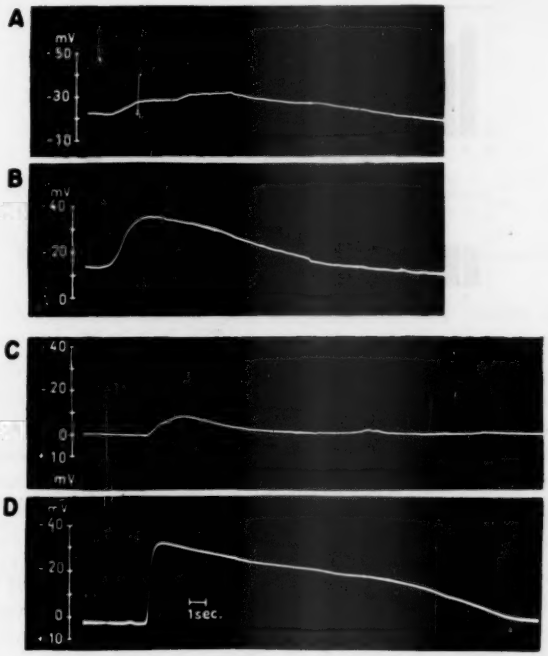

Fig. 4. Microelectrode experiments on perfused glands. A and B show intracellular record from gland cells with secretory potentials over the outer cell membrane resulting from injection of 1 μg acetylcholine. A was obtained 12 minutes after the start of perfusion with nitrate-saline and B, 11 minutes later, three minutes after exchange to a perfusion fluid containing 100 per cent chloride. C and D were obtained from lumina of sublingual gland cell tubuli, C 10 minutes after the start of perfusion with nitrate-saline and D 10 minutes later, 5 minutes after exchange to perfusion fluid containing 100 per cent chloride.

records are from the same experiments, but not from the same gland cell tubule. Record A was obtained 12 minutes after the start of perfusion with nitrate-saline and record B 11 minutes later during perfusion with chloride-saline. In the lower set of records recording was made from the lumen; record C was obtained during perfusion with nitrate-saline and D from another gland cell tubule after change to a solution containing full chloride. In the nitrate-perfused gland the potential change recorded from the lumen was of the same sign and about the same size as that over the outer membrane. Now it is known that during stimulation

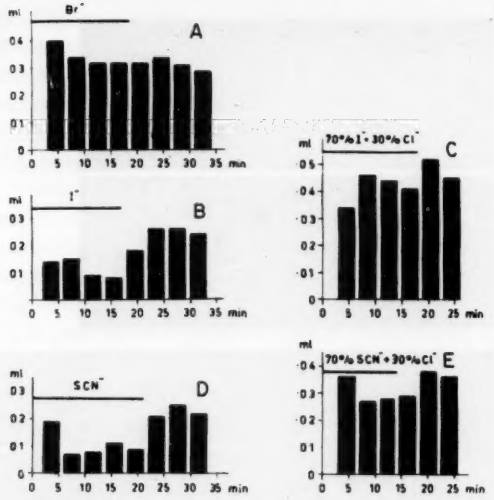

Fig. 5. Secretion in perfused glands. Effect of complete and partial substitution of chloride ion by bromide (A), iodide (B and C) or thiocyanate (D and E).

of the chorda there is also a potential change over the inner membrane (LUNDBERG 1957 a). In order to account for the secretion remaining in the nitrate-perfused gland it could be postulated that this potential change over the inner membrane was the sign of a secretory process that functioned in the absence of chloride ions in the external fluid. If so, we would not have expected any secretory potential over the outer membrane in the nitrate-perfused gland, but a potential change in the positive direction on recording from the lumen. It is obvious that the present findings do not favour this alternative.

Alternatively the secretion as well as the potential change in the nitrate-perfused gland can be accounted for by assuming that, when activated, the outer membrane had a limited capacity for active transport of nitrate ions.

Perfusion experiments have also been made with the use of other monovalent anions. It appears that, when the gland is perfused with a solution with chloride replaced by bromide, secretion is not significantly reduced (Fig. 5 A). However, with iodide or thiocyanate the amounts of saliva on activation with acetylcholine decrease to about the same extent as when the gland is perfused with nitrate-saline (Figs. 5 B, D). With 30 per

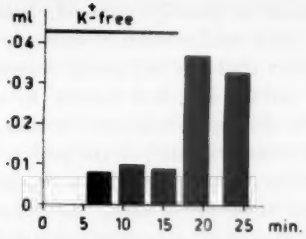

Fig. 6. Decline of secretion in gland perfused with potassium-free saline.

cent chloride there is for both these ions a much smaller decrease of secretion (Figs. 5 C, E), showing that the large depressions of secretion during perfusion by 100 per cent iodide or thiocyanate are entirely or largely due to the absence of chloride ions.

In all the experiments hitherto described the perfusion fluid contained normal amounts of sodium, potassium and calcium. It was observed that secretion failed rapidly if the perfusion fluid contained only sodium chloride. Subsequent experiments proved that potassium but not calcium ions were requisite for maintenance of the gland's secretory ability.

In the experiment illustrated in Fig. 6 secretion during perfusion with potassium-free saline is about 25 per cent of the amount found after exchange to a perfusion fluid containing normal amounts of potassium. In two of the four experiments the failure of secretion was more gradual than in this case. The effect of withdrawal of calcium ion was tested in two experiments and in neither of them was there any impairment of the secretory ability.

Discussion.

The hypothesis, that secretion in the sublingual gland is caused largely by an active transport of chloride ion over the outer gland cell membrane, was formulated on the basis of electrophysiological experiments. The experiments on perfused gland give further evidence supporting this hypothesis.

It is noteworthy that secretion as well as the secretory potential over the outer membrane is normal with no other anions in the external medium than chloride, but that if chloride is replaced by nitrate the amount of secretion decreases to 10—25 per cent of normal and that simultaneously the secretory potential over

the outer membrane is greatly reduced. Since this failure is rapidly reversible with readmission of chloride even in low concentration, it is clear that the failure of secretion is not due to a toxic action of nitrate ion, but merely to the withdrawal of chloride ion from the external medium. It should particularly be noted that the experiments with nitrate-perfused glands virtually exclude the possibility of any considerable "concealed" inward active transport of cations over the outer membrane on activation, for the reason that the small secretory potential remaining in the nitrate-perfused gland has the same sign as the normal secretory potential. It can therefore be concluded that the transfer of cations over the outer membrane during gland cell activity is a passive consequence of the anionic transport, and furthermore that secretion of salt and water normally is caused largely by this active transport of chloride ion over the outer membrane.

The process of active anionic transport shows a high degree of ionic specificity with respect to chloride and nitrate ion, but there is still some secretion left to account for in the nitrate-perfused gland. The electrophysiological experiments make it unlikely that this residual secretion is due to an independent secretory process over the inner gland cell membrane, for the reason that, if so, we would then have expected a potential change in the positive direction on recording from the lumen in the nitrate-perfused gland. The residual secretion as well as the small secretory potential in the nitrate-perfused gland can be accounted for, if we assume that the activated outer membrane has a limited capacity to transport nitrate ions. The transporting mechanism does not distinguish between chloride and bromide, hence there is little difficulty in supposing that nitrate and also the other monovalent anions, iodide and thiocyanate, to some degree may be transported by this ionic pump. However, it is not known if bicarbonate ion will substitute for chloride ion. The resting gland cells will produce CO_2; hence there will be bicarbonate ions in the external medium of the gland cells, and the possibility exists that the residual secretion after withdrawal of chloride ion may be due to active transport of bicarbonate ion. Finally it is possible that the large dose of injected acetylcholine could cause an increased filtration permeability in the gland cell membrane, and that in the nitrate-perfused gland the small secretory potential and the residual secretion are both due to diffusional processes.

The finding that secretion fails when the gland is perfused with potassium-free saline deserves comment. Since the sublingual gland saliva contains about 160 mM sodium against 9 mM potassium, the latter ion plays a relatively small role as a cation passively accompanying the actively transported anion. The depression of secretion in the potassium-free medium does, however, not necessarily imply that potassium ions are of direct importance for the chloride pump. An alternative possibility is indicated by the fact that potassium in different tissues is known to be of importance for the active transport of sodium (cf. reviews by HARRIS, 1954; HODGKIN & KEYNES, 1954; USSING, 1954). In the cephalopod giant axon HODKIN & KEYNES (1955) found that the sodium efflux decreased to one third in potassium-free medium and concluded that sodium efflux and potassium influx are coupled although not very rigidly. There is evidence that, as in other tissues, a high concentration of intracellular potassium is maintained in gland cells (WILLS & FENN, 1936; WILLS, 1941a, b; MANERY, 1954). To maintain this state, which presumably is of importance for the function of the cells, sodium extrusion in the resting cell may be linked with potassium uptake and not take place in the absence of potassium in the external medium. Consequently the reason for the failing secretion in glands maintained in potassium-free medium may be that the cells at rest are not maintained in such a state that they can be properly activated.

Summary.

The sublingual gland of the cat has been found to give normal amounts of saliva in response to acetylcholine or stimulation of the chorda when perfused at 20° C with a saline containing physiological amounts of sodium and potassium and chloride and with chloride as the only anion. On intracellular recording normal secretory potentials are found over the outer membrane with activation.

After replacement of chloride by nitrate in the perfusion fluid the amount of saliva in response to acetylcholine drops to 10—25 per cent of normal and the secretory potential over the outer gland cell membrane is greatly reduced. On readmission of chloride, secretion and the secretory potentials recover promptly. With partial substitution of chloride by nitrate, secretion is about

50 per cent at 10 per cent chloride and virtually normal at 50 per cent chloride.

It is concluded i) that the decrease of secretion and secretory potential in the nitrate-perfused gland is due to the lack of chloride ion in the external medium of the cells; ii) that normal secretion of salt and water is largely caused by an active transport of chloride over the outer gland cell membrane.

Of other anions tested with regard to their capacity to maintain secretion bromide was found to substitute fully for chloride and iodide and thiocyanate in about the same degree as nitrate. The possibility is discussed that the activated outer membrane has a limited ability for transport of these latter ions.

On perfusion with potassium-free saline, secretion is greatly reduced. This finding is discussed in the light of the fact that in other tissues the transport of sodium and potassium is linked.

Acknowledgement.

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Der Off-Effekt im menschlichen Elektroretinogramm.

Von

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Der aus Tierversuchen seit langem bekannte positive Off-Effekt wurde von DODT (1951) auch im menschlichen Elektroretinogramm (ERG) nachgewiesen. Er ist das elektrische Äquivalent für die bei »Off« im Sehnerven auftretende Impulsentladungen und weist analog der a-b-Welle mehrere Phasen auf (HECK 1957), die im Folgenden näher studiert werden sollen.

Methode.

Die Ableitung des ERG, seine Verstärkung und Registrierung, sowie die Reizung des Auges durch Flimmerlicht entspricht der früher beschriebenen Methode (HECK 1957). Bei der Verwendung von intermittierenden Reizen wurde ein gleichlanges Hell-Dunkelintervall gewählt. Die in fast allen Abbildungen durch Synchronisation von Kipp und Fotozelle angewandte Superposition von je 10 gleichen Ableitungen gewährleistete die bestmögliche Ausschaltung von Artefakten. Die benutzten Zeitkonstanten sind in den einzelnen Abbildungen verschieden und deshalb jeweils angegeben.

Untersucht wurden 4 augengesunde Personen im Alter von 28—32 Jahren.

Ergebnisse.

Die Abb. 1/1 zeigt das durch einen Lichtreiz von 0.8 Lux und einer Reizdauer von ca. 1.0 sec ausgelöste ERG nach einer *Dunkeladaptation* von 45 min. Das auftretende Potential ist in seiner Form sehr einfach und besteht aus einer trägen positiven Schwankung, die mit »On« beginnt und bei »Off« abbricht. On- und Off-Antwort haben die gleiche Latenz von ca. 80 msec.

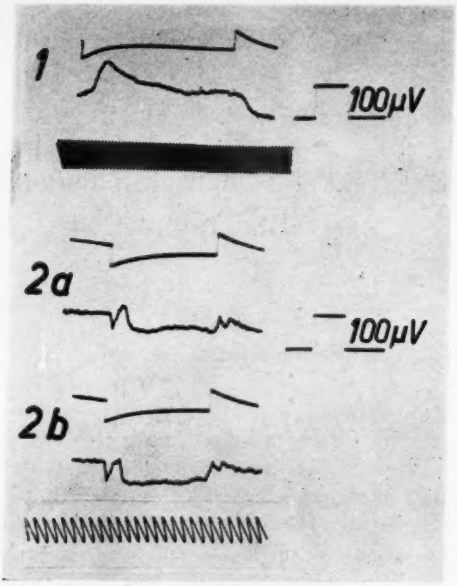

Abb. 1. Menschliches ERG. Über dem ERG Lichtreizmarkierung, beginnend mit »On«. Unter dem ERG Zeitschreibung durch Wechselstrom (50 Hertz).
1: Nach 45 min Dunkeladaptation, Lichtreiz 0.8 Lux, 1 sec.
2a: Nach Helladaptation bei intermittierender Belichtung mit 280 Lux.
2b: mit 480 Lux. Ganz rechts Eichmarken von 100 Mikrovolt. Zeitkonstante in allen Ableitungen 0.7 sec.

Im Gegensatz dazu sieht man in der Abb. 1/2a und 2b das komplexere ERG nach einer *Helladaptation* durch intermittierende Belichtung mit 280 bzw. 480 Lux. Es beginnt mit einer tiefen a-Welle, gefolgt von einer 3-zackigen b-Welle. Danach sinkt das Potential spontan bis zum tiefsten Punkt der a-Welle und bleibt solange unter der isoelektrischen Linie, bis beim Erlöschen des Lichtes ein *doppelter positiver Off-Effekt* auftritt. Dieser besitzt im Gegensatz zu der Off-Schwankung nach Dunkeladaptation eine auffallend kurze Latenz von ca. 18 msec bei 280 Lux, die der Latenz der a-Welle entspricht.

Einzelheiten der Off-Effekte werden bei grösserer Verstärkung und verschiedener Lichtintensität in der Abb. 2 sichtbar. Die Schwelle für den 1. Off-Effekt liegt bei 20 Lux, die für den 2. bei 40 Lux. Die in ihrer Form fast gleichen Effekte weisen im absteigenden Schenkel je eine kleine positive Zacke auf.

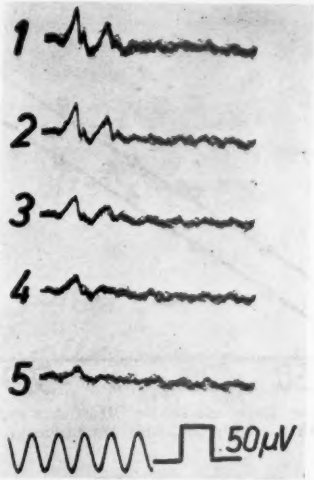

Abb. 2. In jeder Ableitung 10-fach superponierter Off-Effekt. Der Kipp beginnt mit »Off«. 1—5 abnehmende Lichtintensität (280, 140, 70, 40, 20 Lux), Flimmerfrequenz 2 Hertz. Zeitmarke 50 Hertz, Eichmarke 50 Mikrovolt. Zeitkonstante 70 msec.

Der Einfluss der Lichtintensität geht aus der Abb. 3 hervor die zeigt, dass beide Off-Effekte linear mit dem Logarithmus der Lichtintensität an Amplitude zunehmen.

Im folgenden wurde der Doppeleffekt der Off-Antwort näher untersucht und zu diesem Zweck mit der unter gleichen Bedingungen abgeleiteten a-b-Welle verglichen. In der Abb. 4 sind »On« und »Off« so untereinander gesetzt, dass der Kipp jeweils mit dem Lichtwechsel beginnt. Dabei fällt die bereits erwähnte Gleichheit der Latenz von a-Welle und erstem Off-Effekt besonders auf. Ferner ist die in der aufsteigenden b-Welle vorhandene erste Zacke spiegelbildlich im absteigenden Teil des ersten Off-Effektes zu sehen. Die höchste Erhebung der b-Welle entspricht der tiefsten Stelle zwischen 1. und 2. Off-Effekt. Der zweite Off-Effekt wiederum fällt zeitlich mit einer Zacke zwischen dem letzten und vorletzten »hump« der b-Welle zusammen.

Eine noch grössere Verstärkung als in der Abb. 4 liess deutlich erkennen, dass alle Phasen des On-Effektes, wenn auch etwas unterschiedlich in ihrer Amplitude, spiegelbildlich im Off-Effekt gefunden werden.

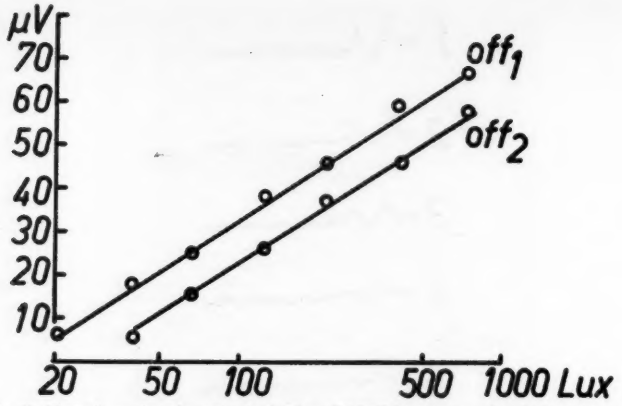

Abb. 3. Verhalten der Amplitude beider Off-Effekte gegen die Lichtintensität. Abszisse: Logarithmus Lux; Ordinate: Amplitude in Mikrovolt.

In einer früheren Untersuchung fanden wir (HECK und RENDAHL 1957), dass sich in der b-Welle durch selektive Adaptation einzelne Phasen unterdrücken lassen. Dass dies im gleichen Mass auch für den Off-Effekt gilt, zeigt die Abb. 4/2, wo mit einer Intensität von 280 Lux bei einer ständigen Grundbelichtung von 100 Lux geflimmert wurde, einem Hellintervall also ein weniger helles Intervall folgt. Hierdurch wird der erste Teil der b-Welle wenig beeinflusst, während ihre beiden letzten Zacken fehlen. Analog dazu ist das Verhalten des Off-Effektes, dessen zweite Phase verschwindet.

Besprechung der Ergebnisse.

Dem positiven monophasischen ERG des Menschen mit schwachen Lichtreizen nach Dunkeladaptation steht das komplexe polyphasische ERG nach Helladaptation gegenüber. Es fällt vor allem das Auftreten eines negativen Potentials durch die Helladaptation auf, analog den tierexperimentellen Befunden (Zusammenfassung: GRANIT 1947). Aus der Abb. 1 erkennt man, dass diese Negativität ebensolange anhält wie der Lichtreiz. Die On-Antwort gleicht hier ganz dem von SCHUBERT und BORNSCHEIN (1952) zuerst beschriebenen Einzelreiz-ERG eines congenital Nachtblinden. Dies ist verständlich, da ja die Helladaptation

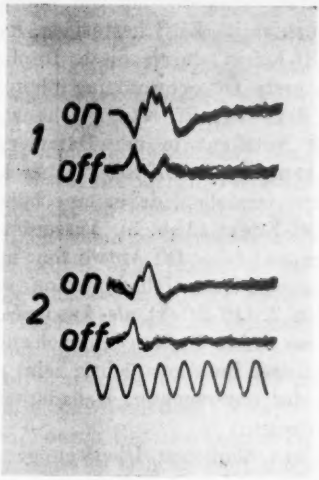

Abb. 4. 1: On- und Off-Effekt bei Flimmerlichtreizung mit 280 Lux, 2 Hertz. 10 superponierte ERG in jeder Ableitung. Der Kipp beginnt jeweils mit dem Lichtwechsel.
2: Wie bei 1, jedoch unter ständiger Grundbelichtung von 100 Lux (sodass einer Hellphase eine weniger helle Phase folgt). Zeitmarke 50 Hertz. Zeitkonstante 70 msec.

durch das Flimmerlicht auch bei Gesunden die skotopische Aktivität reduziert und photopische Komponenten hervortreten (Dodt 1951).

Der am Ende des Lichtreizes auftretende *doppelte positive Off-Effekt*, der kürzlich von Best und Bohnen (1957) sowie Heck (1957) beschrieben wurde, stellt nach der Abb. 4 das *Spiegelbild der On-Antwort* dar und lässt bei grösserer Verstärkung analog der mehrzackigen b-Welle zwei weitere kleine positive Schwankungen erkennen (siehe auch Abb. 2/1). Die sich in On- und Off-Effekt entsprechenden Phasen weisen ferner gleiche Latenz auf und werden durch Variation des Lichtreizes gleichsinnig beeinflusst. Es ist darum zu vermuten, dass »On« und »Off« durch die gleichen Prozesse gebildet werden, wie es bereits im Tierversuch von Granit und Mitarbeitern festgestellt wurde.

Seitdem Adrian (1945) im menschlichen ERG (dem Befund von Granit und Munsterhjelm 1937 beim Frosch entsprechend) je eine photopische und skotopische b-Welle beschrieb, die er als Erregung der Zapfen und Stäbchen deutete, liegen zahlreiche

Arbeiten vor, die das Vorhandensein von photopischen und skotopischen Komponenten im ERG bestätigen. Es liegt nahe, auch den doppelten Off-Effekt durch diese Duplizität zu deuten. Danach wäre die erste Off-Schwankung photopisch, die zweite skotopisch. Mit dem Begriff »skotopisch« wird konventionell die Funktion der Netzhaut in der Dämmerung ausgedrückt. In diesem Sinne kann der 2. Off-Effekt aber keine reine skotopische Komponente darstellen, da er eine höhere Schwelle aufweist als der 1. Off-Effekt (Abb. 3). Ausserdem konnten wir in allen Untersuchungen zwei Off-Antworten nachweisen, selbst bei einer vorausgegangenen Helladaptation von maximal 800 Lux. Sieht man den 2. Off-Effekt als Ausdruck der Stäbchenerregung an, so muss dieses System demnach auch noch bei der genannten Lichtintensität funktionstüchtig sein; mit anderen Worten, es kann bei der untersuchten Helladaptation keine »Stäbchenblindheit« vorliegen.

GRANIT prägte aus ähnlichen Überlegungen auf Grund tierexperimenteller Befunde den Begriff der sog. *»cone-like rods«*, die er wie folgt charakterisiert: »The cone-like rods are light resistant, recover quickly to a semi-stationary level of sensitivity of relatively high threshold and probably contain a visual purple which is chemically slightly different from the usual form and is capable of mediating responses in the photopic state. These are the only rods which remain active after light adaptation. The real or "ideal" rods are only able to influence the response (either of the whole retina or of one nerve fibre) after a period of dark adaptation long enough to allow sufficient visual purple to be formed. . . .« (Sensory mechanisms of the retina 1947 Seite 254). Durch diese »Cone-like rods« lässt sich auch der positive off-Effekt der reinen Stäbchen-Retina des Gecko (DODT und HECK 1954) deuten, der in dieser Form sonst nur bei Anwesenheit von Zapfen beobachtet wird.

Bei BORNSCHEIN und SCHUBERT (1953) sowie GOODMAN und ISER (1956) finden sich im ERG congenital-nachtblinder Patienten ebenfalls doppelte positive Off-Effekte, die trotz der dort angewandten geringeren Potentialverstärkung deutlich erkennbar sind, von den Verfassern aber nicht näher beschrieben wurden. GOODMAN und ISER wiesen darauf hin, dass in der a-Welle ihres Hemeralopen auch eine skotopische Komponente vorhanden war. Das oben erwähnte spiegelbildliche Verhalten der On- und Off-Antwort legt den Schluss nahe, dass entsprechend auch im Off-

Effekt skotopische Komponenten im Sinne von »Cone-like rods« auftreten. Dafür könnte auch die Tatsache sprechen, dass congenital Nachtblinde keine Gesichtsfeldeinschränkungen aufweisen. Histologische Untersuchungen solcher Netzhäute liegen, soweit uns bekannt, nicht vor.

Schliesslich muss man die Beteiligung von Farbkomponenten am Off-Effekt in Erwägung ziehen. So beschrieben Goto und Toida (1954) beim Frosch eine mehrphasige d-Welle bedingt durch unterschiedlich schnelle Erregungsausbreitung einzelner Farbkomponenten, wobei eine Rotphase vor Grün und Blau auftrat. Gleiche Ergebnisse erbrachte die Methode von Motokawa und seiner Schule (1949—56) sowie die Untersuchungen Donner's (1950) mit Hilfe der Mikroelektrode.

Auch in der polyphasischen b-Welle des menschlichen ERG finden sich offenbar farbspezifische Komponenten, wie Heck und Rendahl (1957) durch Untersuchungen Farbenblinder zeigen konnten. Dabei handelte es sich lediglich um Veränderungen an den letzten beiden »humps« der b-Welle. Da diese zeitlich und in ihrem Verhalten gegen Adaptationsänderungen dem 2. Off-Effekt entsprechen (Abb. 4), ist zu vermuten, dass auch nur der 2. Off-Effekt farbspezifische Eigenschaften besitzt. Demgegenüber scheinen 1. Off-Effekt und 1. Teil der b-Welle mehr der Ausdruck für eine Helligkeitserregung zu sein, wie auch subjektiv vor der Farberkennung eine Helligkeitsempfindung wahrgenommen wird.

Die heutigen Untersuchungsergebnisse reichen nicht aus, um eine Deutung aller elektrischen Erscheinungen am Auge zu geben. Erstaunlich bleibt jedenfalls die Feststellung, dass mit dem Wechsel des Adaptationszustandes eine völlige Veränderung des ERG einhergeht, die sich nicht allein durch die Verminderung der Sehpurpurmenge erklärt, sondern vielmehr als Ausdruck einer Umstimmung der gesamten Netzhaut durch die Belichtung angesehen werden muss.

Zusammenfassung.

Nach Helladaptation findet man im menschlichen ERG bei intermittierender Belichtung neben einer komplexen On-Antwort, die dem Einzelreiz-ERG von congenital Nachtblinden entspricht, einen doppelten positiven Off-Effekt. »On« und »Off« weisen spiegelbildliches Verhalten auf. Die Abhängigkeit des Off-Effektes

vom Adaptationszustand und von der Lichtintensität wird untersucht, und das Zustandekommen seiner doppelten Konfiguration diskutiert.

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Messungen von cerebralen Kreislaufzeiten am Menschen und ihre Beziehung zur Gehirndurchblutung.

Von

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Untersuchungen über Kreislaufzeiten des Gehirns liegen nur in geringem Umfange vor. An Katzen haben WOLFF und BLUMGART 1929 solche Messungen vorgenommen. Am Menschen sind die meisten derartigen Untersuchungen zu röntgendiagnostischen Zwecken an Angiogrammen vorgenommen worden, wobei im allgemeinen nur gewisse Teile des Gehirnkreislaufs studiert wurden (s. ausführliche Darstellung bei GREITZ 1956).

Injektionsmethoden nach dem Indikatorverdünnungsprinzip sind von NYLIN und BLÖMER (1955), hauptsächlich zur Durchblutungsmessung, und von GREITZ (1956) für die Bestimmung von Kreislaufzeiten des ganzen Gehirns benutzt worden.

Vergleichende Untersuchungen zwischen Kreislaufzeit und Durchblutung des Gehirns liegen, abgesehen von einer kurzen Bemerkung bei LASSEN und MUNCK (1955, S. 36) nicht vor.

In der vorliegenden Arbeit wird über eine Untersuchung von cerebralen Kreislaufzeiten am Menschen mit besonderer Berücksichtigung ihrer Beziehung zum Durchströmungsvolumen berichtet.

Material.

Die Untersuchungen wurden in der Fortsetzung der von BROBEIL, HÄRTER, HERRMANN und KRAMER (1954) publizierten Untersuchungs-

serie durchgeführt. Die hier beschriebenen Messungen wurden an 61 Patienten aus der neurologisch-psychiatrischen Universitätsklinik in Marburg a.d. Lahn vorgenommen. Das Material bestand aus 48 Männern und 13 Frauen im Alter von 18—67, durchschnittlich 46 Jahren. Es befanden sich darunter 8 Fälle von Hirnatrophie, 6 von Enzephalitis mit Folgezuständen, 5 von Schizophrenie, 4 von multipler Sklerose, 4 von Epilepsie, je 3 von Depressionszuständen, Mb. Parkinson und Psychopathie; im übrigen einzelne Fälle von peripherer Nervschädigung, amyotrophischer Lateralsklerose, Lues, Hirntumor u.a. Arterielle Hypertonie lag in 17, ausgesprochene Demenz in 4 Fällen vor. Herz- und Blutkrankheiten waren nicht repräsentiert.

Methoden.

Die Patienten wurden vormittags in ruhendem, nüchternem Zustand untersucht. Die Gehirndurchblutung wurde nach der Kety'schen Methode (KETY und SCHMIDT 1945, 1948 a, KETY 1948) gemessen (vgl. BROBEIL u. Mitarb. 1954), die Kreislaufzeit durch kontinuierliche Registrierung der Sauerstoffsättigung des arteriellen und venösen Blutes mit dem in der vorhergehenden Arbeit (NILSSON 1957) angegebenen oximetrischen Verfahren. Bei der Zeitbestimmung wurde angestrebt, die Messung möglichst ohne Mitwirkung des Patienten auszuführen, um eventuelle Störungen durch Körperbewegungen, Pressen u. dgl. zu vermeiden, und um auch nicht-mitwirkungsfähige Patienten untersuchen zu können. Die Zeit vom Umschalten auf eine Atemgasmischung mit verändertem Sauerstoffgehalt bis zum Auftreten der Sättigungsveränderung in der Ohrkurve (Lunge — Ohr — Zeit) wird dadurch auch von der augenblicklichen Atmungsintensität des Untersuchten abhängig. Sie ist hier nicht näher analysiert worden.

In einem Teil der Serie wurde Gehirndurchblutung und Kreislaufzeit nach einer Pause von 20—30 min nach der ersten Bestimmung zum zweiten Mal gemessen unter Verwendung eines Gasgemisches mit 5 % Kohlendioxyd. Für die Stickstoffgaben bei der Kreislaufzeitmessung wurde dabei eine Mischung von 5 % CO_2 in N_2 gebraucht.

Für die Feststellung der Kreislaufzeit wurden jedesmal 2—5 Messungen ausgeführt und als Ergebnis der Mittelwert hiervon unter Nichtbeachtung eventuell schlecht ablesbarer Werte betrachtet. Die Kreislaufzeitmessung erfolgte regelmässig in unmittelbarem Anschluss an die Messung der Gehirndurchblutung.

Ausser den oben genannten und den gewöhnlichen klinischen Untersuchungen wurden in fast allen Fällen auch Röntgenuntersuchungen in Form von Carotis-Angiographie und Encephalographie vorgenommen. Eine nähere Analyse der hierbei gewonnenen Befunde und ihre Beziehung zur Gehirndurchblutung wird an anderer Stelle publiziert.

Der arterielle Blutdruck wurde auskultatorisch zu bestimmten Zeitpunkten während der Messung der Gehirndurchblutung bestimmt, bei der Kreislaufzeitmessung meistens sowohl zwischen den Stickstoffgaben wie auf deren Höhe im Anschluss an die Zeitmessung.

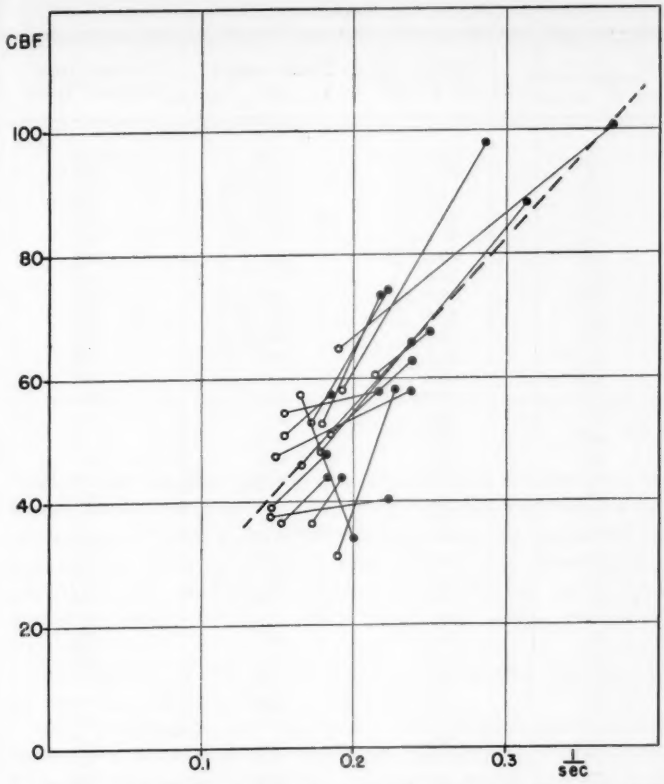

Abb. 1. Beziehung zwischen Durchblutungswert nach Kety (CBF) in ml/100 g · min und inversem Wert der Kreislaufzeit vor und während Einatmung von 5 % CO_2. Offene Kreise: Werte ohne CO_2. Punkte: Werte mit CO_2. Die ausgezogenen Linien verbinden Messungen am gleichen Patienten. Die Regressionslinie sämtlicher Werte (gestrichelt) hat die Gleichung

$$y = 272\,x + 1{,}2$$

Resultate.

Abhängigkeit von der Gehirndurchblutung. Die Resultate der Messungen sind in der Tabelle dargestellt. Es stellt sich heraus, dass die Kreislaufzeit und die Gehirndurchblutung fast unabhängig voneinander variieren. Da eine lineare Abhängigkeit von der Durchblutung eher für die lineare Ström-

Tabelle.

Nr.	CBF ml/100 g · min	Kreislaufzeit sec	Kreislaufzeit inverser Wert
1	33.3	7.8	0.128
2	57.1	6.0	167
3	31.4	6.3	159
4	47.4	5.9	170
5	45.9	5.7	175
6	60.9	6.2	161
7	29.8	7.2	139
8	48.8	8.6	116
9	47.6	5.1	196
10	38.4	5.2	192
11	22.3	5.9	170
12	36.5	5.8	172
13	46.7	3.3	300
14	39.8	5.1	196
15	48.8	5.2	192
16	39.9	6.2	161
17	90.1	4.3	232
18	41.8	4.5	222
19	53.0	5.8	172
20	48.7	4.8	208
21	54.8	6.5	154
22	46.3	6.1	164
23	47.6	6.8	148
24	60.7	4.7	213
25	41.3	6.3	159
26	59.8	4.8	208
27	48.3	5.6	179
28	41.0	7.4	135
29	36.5	6.6	152
30	40.3	6.7	149
31	51.0	6.5	154
32	57.2	5.6	179
33	31.3	5.3	189
34	47.1	4.8	208
35	34.3	6.1	164
36	39.8	5.4	185
37	38.0	6.4	156
38	39.2	4.5	222
39	69.8	7.3	137
40	39.3	6.9	145
41	32.9	5.1	196
42	50.9	5.6	179
43	37.7	6.9	145
44	51.1	5.4	185
45	52.7	5.6	179
46	29.6	6.0	167
47	58.3	5.2	192
48	65.0	5.3	189
49	34.5	5.1	196

Nr.	CBF ml/100 g · min	Kreislaufzeit sec	Kreislaufzeit inverser Wert
50	69.0	4.0	0.250
51	40.0	6.4	156
52	43.3	5.3	189
53	53.8	10.6	094
54	44.7	3.8	263
55	32.7	4.8	208
56	51.0	8.0	125
57	38.9	6.5	154
58	50.2	4.2	238
59	68.6	4.9	204
60	52.7	7.8	128
61	50.0	4.4	227

ungsgeschwindigkeit als für die Kreislaufzeit zu erwarten ist, wurde die Korrelation zwischen den Durchblutungswerten und den inversen Werten der gemessenen Zeiten, als Ausdruck für die lineare Geschwindigkeit, berechnet. Der Korrelationskoeffizient für die Korrelation zwischen Volumen- und Lineargeschwindigkeit beträgt $+0.21$ und ist nicht signifikant ($p > 0.05$).

Der Mittelwert der Kreislaufzeiten im ganzen Material ist 5.7 sec, sein mittlerer Fehler $\pm$ 0.2 sec.

Eine *Korrelation* der hier gemessenen Kreislaufzeiten *zum Alter oder Blutdruck* der Patienten bestand im vorliegenden Material ebensowenig wie bei GREITZ in bezug auf die nach seiner standardisierten Methode gemessenen angiographischen Kreislaufzeiten.

Einfluss von Kohlendioxyd. An 17 Patienten sind Werte auch unter Einwirkung von 5 % Kohlendioxyd erhalten. Die Abb. 1 stellt die Resultate der Durchblutungs- und Kreislaufzeitmessungen vor und während der CO_2-Einatmung dar. In der Abb. 3 sind Beispiele einer Originalkurve wiedergegeben. Die CO_2-Einatmung bewirkt eine signifikante Erhöhung der Lineargeschwindigkeit (Zunahme von $\frac{1}{\text{sec}}$ im Mittel 0.064, $p < 0.001$). In diesem Fall ist eine deutliche Abhängigkeit zwischen Durchströmung und Lineargeschwindigkeit vorhanden, vor allem in bezug auf die Werte während der Kohlen-

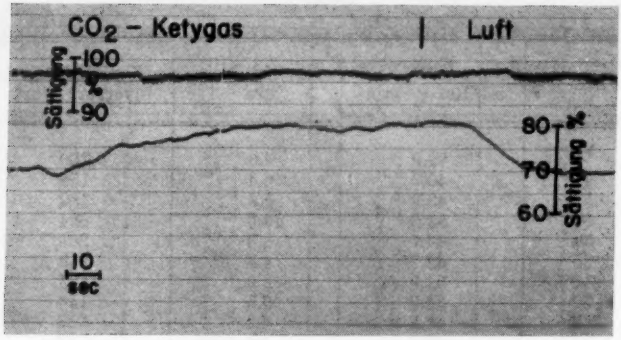

Abb. 2. Verlauf der Arterien- und Venensättigung während kurzdauernder Atmung von 5 % CO_2. Obere Kurve: Ohroximeter, Eichung links. Untere Kurve: Cuvetteneinheit, angeschlossen an den Bulbus jugularis, Eichung rechts. Die Atmung des CO_2-haltigen Gasgemisches fängt am linken Rande der Abbildung an

dioxydatmung. Der Korrelationskoeffizient für sämtliche dargestellten Werte ist $+0.80$ ($p < 0.001$).

Zeitfaktor der Kohlendioxydwirkung. Da die Einatmung von 5 % Kohlensäure keine Veränderung der Sauerstoffaufnahme des Gehirns herbeiführt (KETY und SCHMIDT 1948 b, NOVACK u. Mitarb. 1953), kann man die dabei auftretenden Änderungen der arteriovenösen Sauerstoffsättigungsdifferenz direkt als Ausdruck der veränderten Blutdurchströmung auffassen. Die oximetrische Registrierung bietet dadurch die Möglichkeit, die Zeitverhältnisse bei Anfang und Ende der CO_2-Wirkung zu studieren. Wie in der Abb. 2 veranschaulicht, fängt die dilatierende Wirkung der Kohlensäure nach etwa 15 sec an und erreicht nach ungefähr 1 min ihr Maximum. Das Abklingen verläuft schneller, so dass der Ausgangswert etwa 1/2 min nach dem Aufhören der CO_2-Atmung erreicht ist.

Das Material besteht, wie eingangs erwähnt, aus neurologisch-psychiatrischen Fällen. Als Beispiel einer ausgesprochenen Kreislaufreaktion kann ein im Material nicht berücksichtigter Patient angeführt werden, bei dem während der Punktion ein Schockzustand auftrat mit einem Blutdruckfall von 175/115 mm Hg auf etwa 100/75 mm Hg. Die in diesem Zustand ausgeführte einzige Kreislaufzeitbestimmung gab als Resultat 13.9 sec, die längste überhaupt gemessene Zeit. Ein hierzu gehöriger Durchblutungswert wurde nicht ermittelt.

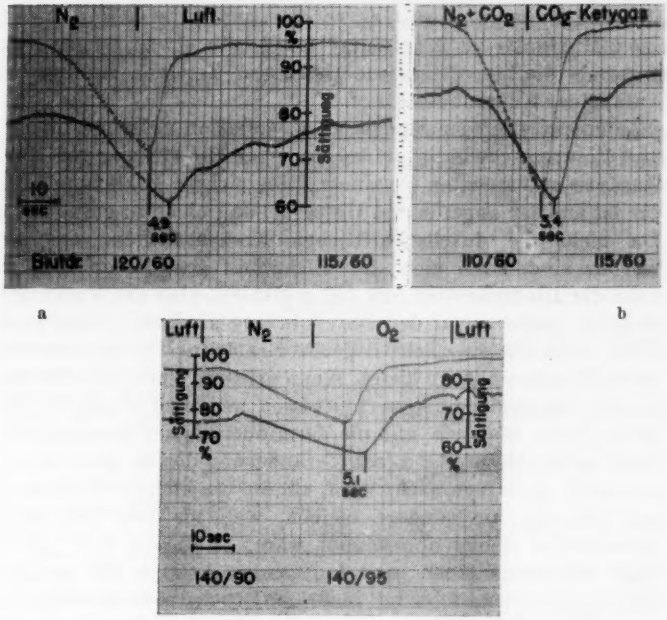

Abb. 3. Sättigungsverlauf während kurzdauernder Stickstoffatmung. a) und b) bei derselben Person vor bzw. während Einatmung eines Gasgemisches mit 5 % CO_2 (CBF = 58.3 bzw. 98.6 ml/100 g · min). Obere Kurve: Ohroximeter. Untere Kurve: Cuvettenoximeter, angeschlossen an den Bulbus jugularis. Eichung gemeinsam für beide Kurven. c) Messung ohne CO_2 bei einem Patienten mit niedriger Durchblutung (CBF = 34.5 ml/100 g · min).

Diskussion.

Messungen, die mit den hier beschriebenen direkt verglichen werden können, sind in der Literatur sehr spärlich beschrieben. GREITZ (1956) hat am Menschen direkt die Zeit A. carotis — V. jugularis int. durch Injektion von einer radioaktiven Substanz in die A. carotis bestimmt. Da er diese Untersuchung als Verifikation einer angiographischen Zeitmessmethode ausführte, hat er keine nähere Auswertung der Resultate vorgenommen. In seinem Diagramm 7 liegen die Werte zwischen 5 und 10.5 sec, mit einem Mittelwert bei etwa 7 sec. Der Unterschied gegenüber den hier mitgeteilten Werten erklärt sich wahrscheinlich ausser durch eventuelle Verschiedenheiten im

Material dadurch, dass GREITZ die „häufigste" Kreislaufzeit (von Maximum zu Maximum) misst, die etwas länger sein muss als die hier gemessene „kürzeste" Zeit von Anfang bis Anfang der Kurvenausschläge.

Interessant ist der Mangel an Korrelation zwischen Linear- und Volumgeschwindigkeit bei den ohne Einwirkung von Kohlensäure untersuchten Personen. Eine sichere Erklärung hierfür ist aus der angeführten Untersuchung nicht zu entnehmen. Da nicht die durchschnittlichen Kreislaufzeiten gemessen worden sind, kann nicht mit Sicherheit geschlossen werden, dass die Blutquantität pro 100 g Gehirn, also die Vaskularisierung, proportional der Durchblutung abnimmt, wenn auch diese Annahme die nächstliegende Erklärung für die beobachteten Zusammenhänge bietet. Wenn grössere Verschiedenheiten in den Durchströmungsverhältnissen zwischen einzelnen Gehirngebieten bestehen, und die Verminderung der Gesamtdurchblutung hauptsächlich durch Veränderung der langsam durchströmten Teile verursacht wird, könnte die kürzeste Kreislaufzeit deshalb unverändert bleiben, weil sie von den nicht veränderten Gebieten bestimmt wäre. In diesem Fall würde man allerdings einen sehr langsamen Anstieg der venösen Sättigung in den Fällen mit niedriger Durchströmung erwarten, was nicht regelmässig der Fall ist (vgl. Abb. 3 c).

Die kurze Bemerkung von LASSEN und MUNCK (1955) über die nur sehr kleine Veränderung der Kreislaufzeit bei grossen Variationen in der Gehirndurchblutung steht mit dem hier erhobenen Befund im Einklang. Die hohen Zeitwerte dieser Verfasser (9—15 sec) werden wahrscheinlich durch die Eigenheiten der Methode erklärt (vgl. NILSSON 1957).

Bei den durch Kohlendioxydatmung hervorgerufenen Durchblutungssteigerungen steigt die lineare Geschwindigkeit in annähernd direkter Proportion. Bei diesen schnellen Umstellungen wird man also mit kleineren Veränderungen der Vaskularisation bzw. geringeren Unterschieden in der Reaktionsbereitschaft der individuellen Gehirngebiete zu rechnen haben.

Zusammenfassung.

An 61 neurologisch-psychiatrischen Patienten wurden Gehirndurchblutung und cerebrale Kreislaufzeit unmittelbar nacheinander gemessen. Der Mittelwert der (kürzesten) Kreislaufzeit war 5.7 ± 0.2 sec.

Es bestand keine signifikante Korrelation zwischen Gehirndurchblutung und linearer Strömungsgeschwindigkeit des Blutes, ausgedrückt durch den inversen Wert der Kreislaufzeit.

Die Einatmung von 5 % CO_2 erhöhte die Lineargeschwindigkeit signifikant. Während der CO_2-Atmung bestand eine starke positive Korrelation zwischen Durchblutung und Lineargeschwindigkeit.

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Oxygen Consumption of the Normal and Denervated Submaxillary Gland in Vitro.

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It has been shown that the addition of secretory drugs to slices of the submaxillary gland causes an increases in oxygen consumption. This indicates that the glandular cells are stimulated by these drugs also in vitro (DEUTSCH and RAPER 1936 and 1938, BROCK, DRUCKREY and HERKEN 1938 and 1939, BROCK, DRUCKREY and LOCH 1943).

Denervation of the submaxillary gland is known to increase the sensitivity of the gland to the secretagogue action of drugs (supersensitivity after denervation). It was thought to be of interest to find out whether an increased sensitivity to drugs could also be demonstrated for the oxygen consumption as measured in vitro.

Methods.

Cats were used as experimental animals.

The denervations were carried out two to three weeks before the acute experiment. In some of the experiments the chorda tympani was cut, in others the superior cervical ganglion was excised in ether anaesthesia under aseptic conditions.

Long continued treatment with either pilocarpine or Hö 9980 was given as described by EMMELIN and MUREN (1952) for pilocarpine (subcutaneous injections of 0.5 mg/kg body weight in the morning and of 1 mg/kg body weight in the afternoon) and by EMMELIN and HENRIKSSON (1953) for Hö 9980 (1 mg/kg body weight once a day subcutaneously). The treatment lasted for periods of 7—8 days for pilocarpine and of 14—18 days for Hö 9980. Hö 9980[1], piperidino-

[1] The drug was kindly supplied by A.B. Webass, Gothenburg.

aethyl-diphenyl-acetamid-hydrochloride, is a long-acting atropine-like drug (for ref. see EMMELIN and STRÖMBLAD 1957).

The acute experiments were performed as follows:

The submaxillary glands were removed from the animal under ether. The capsule of the gland was stripped off and the hilus cleaned with fine scissors; these procedures were found to promote substantially the subsequent handling of the organ. The glands were weighed and chopped with the chopper described by MCILWAIN and BUDDLE (1953). They were then suspended in Krebs' Ringer phosphate medium and the volume was adjusted to 8 ml. The suspension was pipetted into the main compartment of Warburg flasks (1.0 ml in each). In addition the main compartment contained 1.4 ml of Krebs' Ringer phosphate and 0.3 ml of a 5.6 % solution of glucose. The centre well contained a filter paper soaked with 0.3 ml N KOH, the side bulb the drugs to be tested (in a volume of 0.3 ml). The drugs used were dissolved in distilled water after neutralization to bromthymol blue. One flask was a control; it contained 0.3 ml of distilled water in the side bulb. The flasks were filled with O_2 and shaken in a water bath at 37° C. A period of 10 min. was allowed for temperature equilibration. Readings were taken every 6 min. After two 6 min. periods, the contents of the side bulbs were tipped into the main compartment and the readings continued, usually for 1 hour. The readings were corrected for changes in a thermobarometer.

Results.

1. Normal submaxillary glands.

Resting tissue. In experiments with organs in vitro it is essential to know to what degree the viability of the organ is maintained under the conditions used. In the present investigation this was checked by using in each experiment a control flask into which distilled water and no drugs were tipped. The pressure changes in the control flask were thus an expression of the viability of the cells in this and the other flasks containing cells from the same organ. From the graphic illustrations it can be seen that the rate of oxygen uptake in the control flask kept fairly constant during the experimental period and showed only a slight gradual decrease. After one hour the rate of oxygen uptake was about 80 per cent of the starting value (the first two 6 min. periods). In some experiments, readings were taken for several hours; it was found that after 3 hours there was still an oxygen consumption corresponding to about 70 per cent of the starting value.

Apart from the constancy of the oxygen uptake during the experimental period it was of interest to know the absolute amount

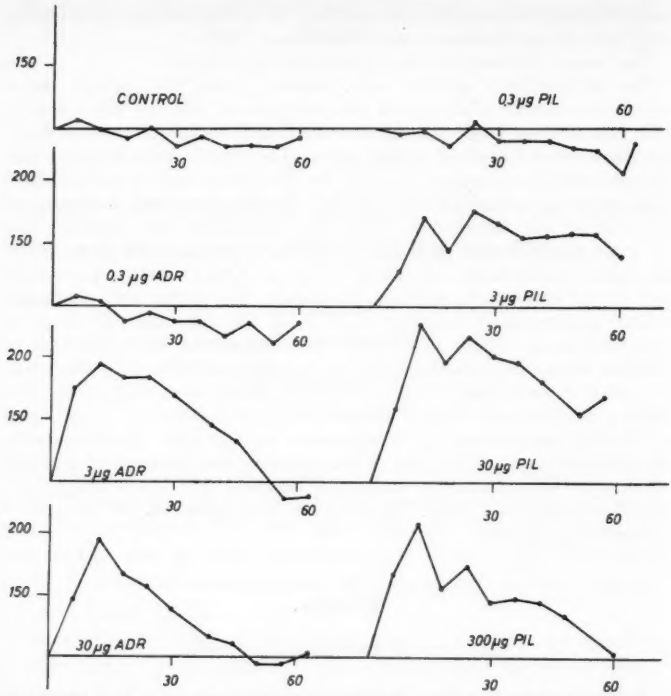

Fig. 1. The figure shows the effect of various doses of adrenaline and pilocarpine on the oxygen consumption of normal submaxillary tissue from one and the same animal.

Ordinate: Oxygen consumption in per cent of starting value (the first two 6 min. periods).

Abscissa: Time in minutes.

of oxygen consumed by the unstimulated tissue. In the present series this was calculated from the readings before tipping in those experiments in which the whole suspension (8.0 ml) was used in 8 flasks. This method of calculation was thought to be the most accurate one, since there were, inevitably, variations in oxygen consumption between the flasks and since no determinations of the amount of tissue in each flask were made. For 27 normal glands from 20 animals the values thus obtained were 1.91 ± 0.056 ml/hour/gram and 1.94 ± 0.114 ml/hour/gland.

Stimulated tissue. The observations of earlier investigators mentioned above that the addition of secretagogue drugs to sali-

vary gland cells in vitro causes an increase in oxygen consumption were fully confirmed. Thus l-adrenaline, l-noradrenaline, pilocarpine, acetylcholine and eserine were found to exert this effect.

The threshold dose of adrenaline or noradrenaline was about 0.3 μg, that of acetylcholine or pilocarpine about 1 μg. With increasing doses the effect increased until a certain level was reached. With a further increase of the doses there was often a decreased effect as reported elsewhere (STRÖMBLAD 1956 d). The maximal effect of adrenaline or noradrenaline was obtained with doses of 10—30 μg, that of pilocarpine or acetylcholine with doses of 30—300 μg. The dose-response relation for adrenaline and noradrenaline can be seen in figs. 3 and 4, respectively. When the maximal increase obtained with adrenaline or noradrenaline was compared, on one and the same gland, with that obtained with acetylcholine or pilocarpine it was found that the two latter substances always produced a bigger effect. In fig. 1 an example of this is shown. The responses to 3 μg and to 30 μg of adrenaline reach the same figures viz. 195 per cent of the resting figure; thus this figure can be taken as the maximal increase obtainable with adrenaline. The response to 30 μg of pilocarpine was 225 per cent of the resting value.

When adrenaline and pilocarpine were given together in doses that gave the maximal effect obtainable with each drug the response was not bigger than with pilocarpine alone.

In some experiments, α-dinitrophenol was used. This substance was found to increase the oxygen consumption of the submaxillary gland. It was thought of interest to compare the maximal increase in oxygen consumption to be obtained using this drug with the increase obtainable with pilocarpine. To get the maximal effect of dinitrophenol it was necessary to work within a very narrow range of doses (fig. 2). The effect of 10 μg and 100 μg was in this experiment much less than that of 30 μg. For different glands the most effective doses varied between 30 μg and 300 μg. Doses of the order of 1 mg invariably caused a rapid decline in oxygen uptake. The maximal increase in oxygen uptake obtainable with dinitrophenol was slightly higher than with pilocarpine; the differences were in the order of 10—20 per cent. In fig. 2 a comparison between dinitrophenol and pilocarpine is also shown. The effects of pilocarpine and dinitrophenol were not additive when the drugs were given in doses causing maximal response with each drug.

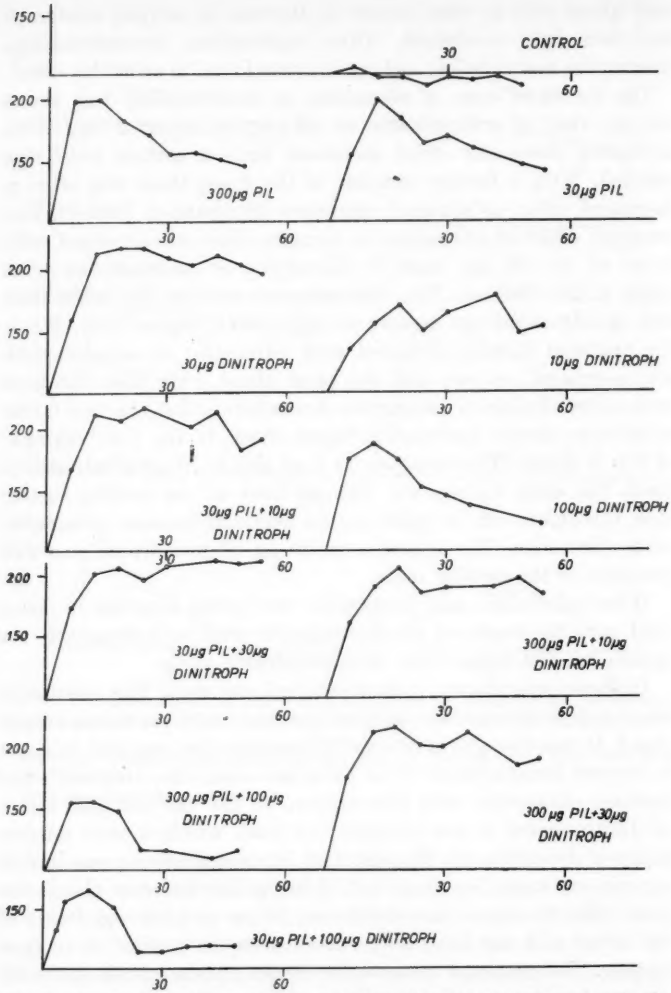

Fig. 2. The figure shows the effect of various doses of dinitrophenol and pilocarpine on oxygen consumption of normal submaxillary tissue from one animal. For further explanations see text and fig. 1.

Eserine was found to cause an increase in oxygen consumption when added in doses between 10 and 1000 µg. Bigger doses were not tried. The effect was slower in onset than with the other

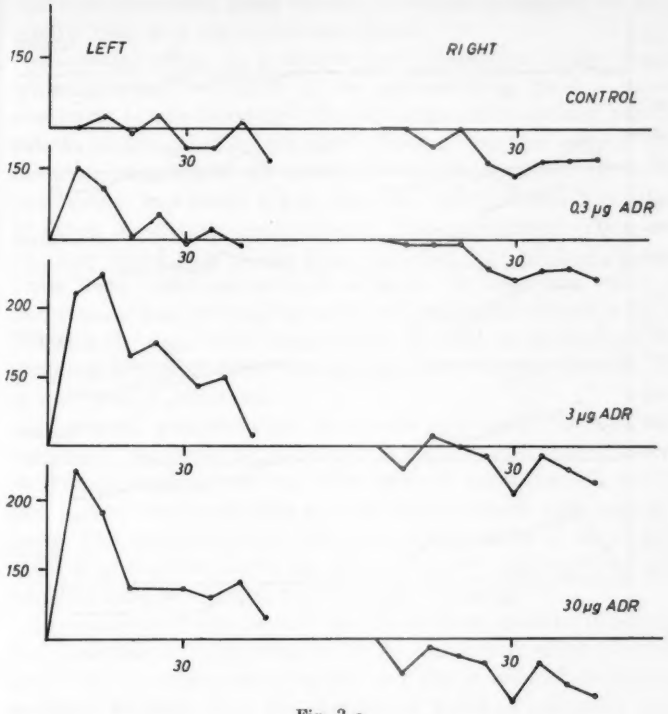

Fig. 3 a.
Comparison between the effect of adrenaline on normal (left) and para- are given to the far right.

substances studied. The oxygen uptake increased gradually and the full effect was often not obtained until 30 min. after the addition of the drug. These findings tally with the conception that acetylcholine is continually released and allowed to accumulate in the presence of eserine.

2. *Parasympathetic denervation.*

The effect on oxygen consumption of previous section of the chorda tympani was studied in 12 animals.

Resting tissue. In 9 out of the 12 experiments, all of the tissue was used in both the denervated and its contralateral gland; as already discussed, this allowed the calculation of the oxygen

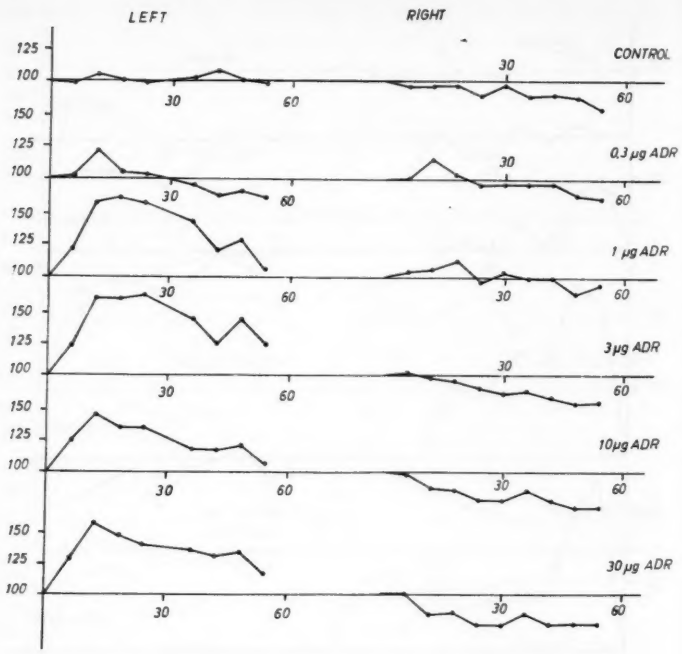

Fig. 3 b.
sympathetically denervated (right) submaxillary tissue. The various doses used Two experiments are shown (3a and 3b).

consumption of the resting tissue. In 8 out of these 9 experiments the oxygen uptake per g was lower in the denervated than in its contralateral, normally innervated gland. If the oxygen consumption of each denervated gland was expressed in per cent of its contralateral gland a value of 82.5 ± 5.82 ($n = 9$) per cent was obtained. The mean value in ml/hour/gram for the denervated glands was 1.58 ± 0.104 ($n = 9$), that for their contralateral glands 1.93 ± 0.095 ($n = 9$). The P value of the difference between 1.58 ± 0.104 for the denervated glands and the corresponding figure of 1.91 ± 0.056 ($n = 27$) for the normal glands, is less than 0.01. If the values were expressed per gland instead of per gram the differences were even more pronounced, since denervation is known to cause a loss in weight. There was no indication,

that in a denervated gland the rate of oxygen uptake fell off more rapidly than in a not denervated gland.

Stimulated tissue. It is known that destruction of the chorda tympani induces regularly in the submaxillary gland a supersensitivity to the secretory effect of drugs and it seemed reasonable to assume, that an increased effect of drugs on oxygen consumption should also be caused by the denervation. However, denervation was found not to increase, but to lessen, the effect of drugs on oxygen consumption. This unexpected effect was regularly found with all the drugs used and with all doses tested. There were variations between animals. In some, the effect of denervation was striking, as in the two examples shown in fig. 3 in which the denervated tissue hardly reacted at all when adrenaline was added. In other animals the effect was less marked, but it was always detectable.

It seemed possible that the denervated tissue had become extremely sensitive to secretagogue agents and that doses of such drugs causing response from normal tissue caused inhibition just as was sometimes seen on normal tissue with very big doses. This possibility was ruled out since a series of very small doses of pilocarpine, 0.001 μg, 0.01 μg and 0.1 μg, was tried and found to have no effect on the denervated tissue.

In conclusion it may be said that denervation caused a lessening, and sometimes a disappearance, of the stimulating effect of secretory drugs on oxygen consumption, and this in spite of the greater secretory response from the denervated gland as compared with that from the normal gland, when the same drugs are tested in vivo.

3. Treatment with pilocarpine.

Long continued treatment with pilocarpine was tried, as it is known that it lowers the sensitivity to secretory drugs of the denervated submaxillary gland to the level of the normal gland. In order to see whether pilocarpine could restore the effect of drugs on oxygen consumption, 5 animals in which the chorda tympani of one side had been cut were treated with pilocarpine.

Resting tissue. All submaxillary glands from the 5 animals were fully used; this allowed estimation of oxygen consumption by the method already described.

The resting values in ml O_2/hour/gram were for the denervated 2.02 and for the normal 2.27; *i. e.* they were higher than for normal untreated glands. The acute experiments were done not earlier than 16 hours after the last injection of pilocarpine, since it is known that the animals never show any secretion 16 hours after the last injection when treated after the schedule used. Nevertheless, in spite of the absence of secretion, the oxygen uptake was higher in the treated than in the untreated animals.

Stimulated tissue. The addition of secretory drugs to glands from the animals treated with pilocarpine caused an increased uptake of oxygen. The increase for various doses was just as great in the denervated as in the normally innervated gland. The similarity in response between the two glands of one and the same animal was found in all 5 cases investigated. Thus treatment with pilocarpine abolished the difference between a denervated and a normal gland with respect to oxygen consumption in vitro.

4. Treatment with Hö 9980.

Long continued treatment with Hö 9980 causes in the submaxillary gland a supersensitivity to the secretory effect of adrenaline, that is even more pronounced than that caused by section of the chorda tympani (EMMELIN and STRÖMBLAD 1957). The effect of treatment with this drug was studied in 8 animals. In 3 of these animals, the chorda tympani was cut on one side.

Resting tissue. The resting oxygen consumption, calculated in ml O_2/hour/gram, was about the same on the denervated as on the normally innervated side (denervated 1.23; normally innervated 1.15). The oxygen uptake per gland was, however, lower on the denervated side (1.04) than on the innervated side (1.42) since the difference in weight between the two glands is seen after treatment with Hö 9980 (cf. STRÖMBLAD 1956 a and b).

The mean value for oxygen consumption in ml O_2/hour/gram for 9 innervated glands from 8 animals treated with Hö 9980 was 1.35 ± 0.087. The P value of the difference between 1.35 ± 0.087 and the corresponding value for normal untreated glands, 1.91 ± 0.056 ($n = 27$) is less than 0.001. The treatment with Hö 9980 had thus lowered the oxygen consumption of resting tissue.

Stimulated tissue. The effect of adrenaline and noradrenaline on glands from animals treated with Hö 9980 was very variable. In

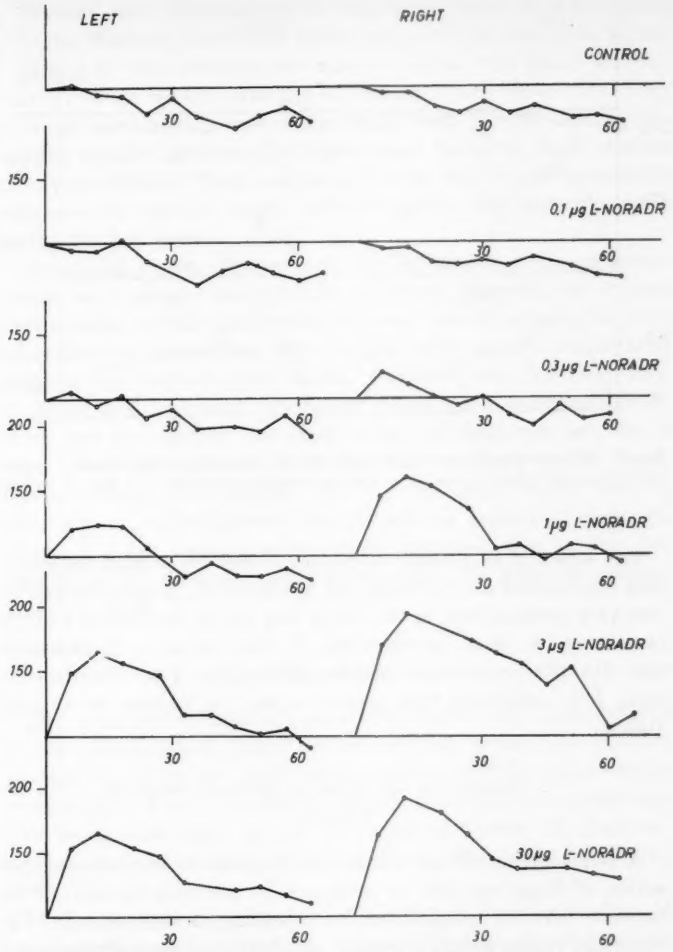

Fig. 4. The effect of various doses of noradrenaline on normal (left) and sympathetically denervated (right) submaxillary tissue.

the 3 experiments where a direct comparison between denervated and innervated gland was possible the denervated gland was found to respond less than the innervated one in 2 cases; in the remaining experiment there was very little effect on any of the glands.

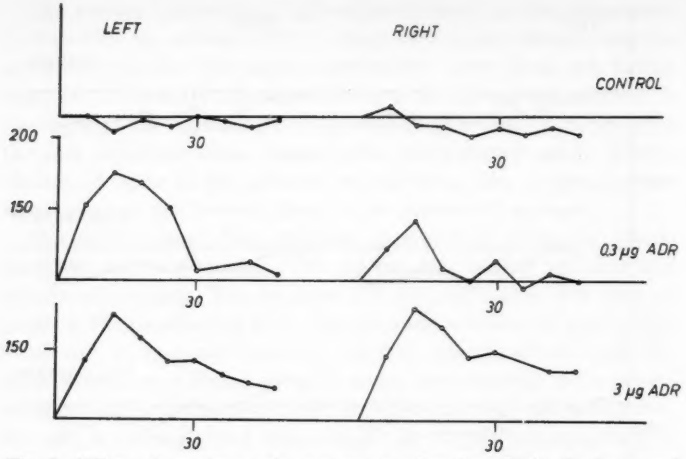

Fig. 5. Effect of cocaine on the response to adrenaline. Right gland removed before and left gland 3 min. after the intracardial injection of 3 mg of cocaine.

The effect of adrenaline or noradrenaline on oxygen consumption were tested on 5 animals not operated on. In one stimulation was very pronounced, in two there was hardly any effect and the remaining two were intermediate. It thus seems as if treatment with Hö 9980 sometimes acts like the section of the chorda tympani, but sometimes this action is less pronounced or entirely absent.

5. *Excision of the superior cervical ganglion. Cocaine.*

A sensitization of the submaxillary gland to the secretagogue action of drugs can also be produced by previous excision of the superior cervical ganglion or by injecting cocaine acutely. The effects of either ganglionectomy or of cocaine were studied each in a series of 4 experiments.

Resting tissue. Ganglionectomy did not seem to influence the resting respiration of the gland. The mean oxygen consumption of the 4 sympathetically denervated glands was 1.78 ml O_2/hour/gram while that of the 4 controls was 1.85. The value of ml O_2/hour/gland was for sympathetically denervated 2.67 and for controls 2.66.

Cocaine was administered in two ways: either it was tipped into the Warburg flask from a side bulb, or 3 mg was given intracardially to the animal some minutes before the gland was removed. When cocaine was tipped into the flask, doses of 0.1 mg to 5 mg were used. Doses of less than 1 mg did not disturb the oxygen uptake whereas the bigger doses caused a rapid decline in oxygen uptake. There was no indication that the intracardially administered cocaine caused any change in the oxygen uptake of the resting tissue.

Stimulated tissue. Excision of the superior cervical ganglion caused an increased effect of the secretory drugs on the oxygen consumption of the gland cells. A given dose of adrenaline, noradrenaline or pilocarpine had a bigger effect on the denervated than on the normal gland tissue. This was true for both submaximal and maximal responses. Thus after ganglionectomy there was not only an increased effect of the drugs but also a higher maximal response. These findings are illustrated in fig. 4. Cocaine, both when tipped into the Warburg vessels from a side bulb and when injected into the animal, caused an increased effect of doses causing submaximal response, but it was not possible to get a bigger maximal response. The effect of cocaine is shown in fig. 5.

Discussion.

The dual nerve supply, parasympathetic and sympathetic, to the submaxillary gland of the cat has raised the problem whether the secretory cells proper have a double nerve supply or whether one set of nerves innervate some cells, the remaining cells being innervated by the other set of nerves (cf. Langenskiöld 1941, Babkin 1950, Emmelin 1954 and Lundberg 1955).

In the present experiments it was found that adrenaline or noradrenaline and pilocarpine or acetylcholine when given in doses causing maximal response are not additive in their effect on oxygen consumption; this indicates that, at least some cells may be stimulated by either of this two groups of substances. There is thus the possibility that a cell may be influenced by both sympathetic and parasympathetic transmitters and that such cells may be the target of both types of transmitter substances released from the two sets of nerves.

The observation on the maximal increase in oxygen consumption obtainable with different drugs merits some consideration. The maximal increase obtainable with adrenaline or noradrenaline was less than with pilocarpine indicating either that adrenaline and noradrenaline respectively could not stimulate all cells or that each cell could not be maximally stimulated by adrenaline or noradrenaline. Which of these possibilities is correct cannot be decided by the present experiments, but it is of interest to note that observers on the innervation of submaxillary glands agree that there are cells that are not innervated by the sympathetic; it is possible that cells not so innervated are not stimulated. A comparison between the maximal effect obtainable with pilocarpine and with dinitrophenol showed that pilocarpine was almost as effective as dinitrophenol; this indicates that pilocarpine can excite all the secretory cells of the gland. The slightly lower figure with pilocarpine may well mean that cells other than secretory cells were present and that these were excited by dinitrophenol but not by pilocarpine.

This investigation was mainly devoted to the study of the effects of secretory drugs on oxygen consumption in denervated tissue and tissue subjected to procedures allied to denervation. It was found that the secretory drugs caused less increase in oxygen consumption in the parasympathetically denervated tissue than in normally innervated tissue. In some cases the denervated tissue showed almost no increase in oxygen consumption after the addition of secretagogue drugs. This was not due to a high basal respiratory activity of the denervated gland since the oxygen uptake of the denervated tissue was less than that of the normally innervated tissue. The results of the experiments were unexpected since it is well known that denervation causes an increased sensitivity of the gland to the secretory effect of drugs. Thus a bigger amount of secretion might be accompanied by a less consumption of oxygen. It is true that there is no indication in the present experiments of a secretory activity, either in the normal or in the denervated tissue, but even if there was, the present experiments may find an interpretation based on what is known about the secretion of the denervated gland. The saliva of the denervated gland differs in its composition from that of the normal gland (cf. BABKIN 1950) and though bigger in volume the extrusion of this saliva may require less work owing to the different composition. In some cases there was almost no increase

in oxygen consumption of the denervated tissue when secretory drugs were added. This may be analogous to the finding of HOKIN and HOKIN (1956), that pancreozymin causes a release of enzyme from the pancreas in vitro without any increase in oxygen consumption.

Another possibility is that the energy requirement for secretion in the denervated gland is not met by increased oxygen uptake but is dependent on anaerobic processes. The work by DEUTSCH and RAPER (1938) indicates that in normal glands the energy for secretion is not provided by anaerobic processes, but it is not known at present if the same holds for the denervated gland.

The decreased effect of secretory drugs on the oxygen uptake of the tissue after denervation seems to be dependent on the lessened activity of the gland caused by denervation: repeated injections of pilocarpine into the animal could prevent the decrease and treatment with an atropine-like drug mimicked to some extent the effect of parasympathetic denervation.

Differences were seen between the effects of parasympathetic and of sympathetic denervation. After excision of the superior cervical ganglion secretory drugs had an increased effect on oxygen uptake. Not only was the response to submaximal doses increased, but also the maximal response was greater after ganglionectomy. It would be of interest to know whether after ganglionectomy a bigger rate of secretion can be obtained in vivo. Similarly cocaine caused an increased effect of submaximal doses, but the maximal response was not increased.

The way in which denervation affects the resting level of oxygen consumption as well as the changes brought about by stimulation is at present unknown, but it does not seem unreasonable to assume that the activity of the respiratory enzymes is influenced by the various procedures adopted. It is known that in mice inactivation of the submaxillary gland by ligating the excretory duct, may reduce the activity of certain respiratory enzymes in the tissue (JUNQUEIRA 1951; FERNANDES and JUNQUEIRA 1952). Furthermore, it is known that parasympathetic denervation of the submaxillary gland causes a decrease of other enzymic systems *e. g.* amine oxidase and cholinesterase, and that treatment with pilocarpine restores the activity of these enzymes (STRÖMBLAD 1956 a and b).

It is known that Hö 9980 causes a decrease in the activity of amine oxidase, but not in the activity of cholinesterase (STRÖMBLAD 1956 a and b). Excision of the superior cervical ganglion seems to increase the activity of amine oxidase in the submaxillary gland (STRÖMBLAD 1956 c). If one or some of the respiratory enzymes limiting the oxygen uptake reacted in the same manner as amine oxidase this could explain the findings of a lessened effect after parasympathetic denervation and after "atropinization". Likewise the effects of both prolonged treatment with pilocarpine and of ganglionectomy would be consistent with such an explanation.

Summary.

Experiments on the oxygen consumption of chopped submaxillary glands in vitro are reported.

1. Secretagogue drugs increased the oxygen consumption of normal glandular tissue.
2. Adrenaline or noradrenaline increased the oxygen consumption less than pilocarpine or acetylcholine. Pilocarpine was almost as effective as dinitrophenol.
3. Previous parasympathetic denervation decreased and sometimes abolished the effect of secretory drugs on oxygen consumption.
4. Prolonged treatment with pilocarpine prevented the disappearance of these effects after parasympathetic denervation.
5. Prolonged treatment with an atropine-like drug induced changes essentially similar to parasympathetic denervation.
6. Previous excision of the superior cervical ganglion or addition of cocaine increased the effect of secretory drugs on oxygen consumption in vitro. Ganglionectomy, but not cocaine, increased the maximal effect obtainable.

Technical assistance was given by Miss ASTA HECTOR.

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Cerebral Sympathetic Vasoconstriction and EEG.

By

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Introduction.

There is reason to believe that cerebral vasomotor mechanisms play an integral rôle in the regulation of the cerebral excitatory state. Evidence has been brought forward that an intimate correlation exists between the EEG and the cerebral vascular resistance. This correlation has been clearly demonstrated experimentally by the fact that the arousal reaction which leads to a general desynchronization of the EEG is accompanied by an increase of cortical blood flow due to a reduction of the cerebral vascular resistance (INGVAR 1955, INGVAR and SÖDERBERG 1956 a and b, 1957, INGVAR 1957). Circulatory adjustments in the brain during changes in its excitatory state are probably elicited through a complex interplay between several factors which depend upon vasomotor innervation as well as upon local humeral events.

In the present investigation we have studied whether the sympathetic innervation of cerebral vessels may exert an influence upon the EEG. In cat preparations, sensitive to arousing stimuli, stimulation and section of the cervical sympathetic nerves was carried out while the EEG and cortical blood flow were recorded continuously. It will be demonstrated that although the sympathetic stimulation gave a small but definite vasoconstriction, and that section gave a vasodilatation, there were no changes produced in the EEG in the large majority of cases. Only under critical conditions did sympathetic vasoconstriction produce slight EEG alterations.

Previous investigations. Since the demonstration of vasomotor nerves to cerebral vessels (STÖHR 1928, PENFIELD 1932) several workers have investigated the vasoconstrictor action in the brain of the cervical sympathetic nerves (FORBES and WOLFF 1928; FORBES, FINLEY and NASON 1933; FINESINGER and PUTNAM 1933; SCHMIDT 1949, 1935, 1950, SCHMIDT and PIERSON 1934, SCHNEIDER and SCHNEIDER 1934, THOMAS 1935, BOUCKAERT and JOURDAN 1949, SCHMIDT and HENDRIX 1938, FOG 1939, LUBSEN 1941, LUDWIGS and SCHNEIDER 1954). Most of the authors mentioned have been aware of the difficulties involved in studying pure cerebral sympathetic vasomotor effects. Stimulation experiments may yield different results in different cerebral regions (SCHMIDT 1934, 1935) and also differences between the grey and white matter (LUDWIGS and SCHNEIDER 1954). The extracranial vessels respond much more powerfully than the intracranial to vasoconstrictor stimulation and this may lead to redistribution of blood in the head (SCHMIDT 1935, LUBSEN 1941, SÖDERBERG and WECKMAN 1957). Cervical sympathetic stimulation is also likely to give changes in the blood pressure (PALME 1943, KEZDI 1954, see below). There is, however, a general agreement from experiments in different species with a number of methods that the cerebral vasoconstriction obtained from cervical sympathetic stimulation is very weak and slow in development in contrast to vasoconstrictor effects in other vascular regions. It is, in general, concluded that "the cerebral vessels are largely withdrawn from the control of vasoconstrictor fibers" (FOLKOW 1955, *cf.* also SCHMIDT 1950).

Methods.

Ten experiments have been carried out in cats. Three were *encéphale isolé* preparations and the remainder were examined under Nembutal anesthesia (40 mg/kg i.p. initially, later prolonged by Pentothal i.v.). The non-anaesthetized *encéphale isolé* preparations as well as those under light anaesthesia were often given Flaxedil (10—15 mg/kg) and maintained with artificial respiration. Blood pressure was measured continuously in the femoral artery with an electromanometer (ELEMA, Solna, Sweden). The temperature of the preparations was checked continuously and heating was applied when necessary.

The cortical blood flow was recorded with the method of INGVAR and SÖDERBERG (1956 a and b, 1957) which entails a measurement of the venous outflow through a poly-ethylene cannula from the superior sagittal sinus after heparinization and elimination of diploic anastomoses. The cranial defect over the exposed sinus was repaired by dental

acrylate cement ("Svedon"), which gave a watertight cranioplasty. Blood drops coming out from the cannula in the sinus gave a signal to an electronic interval recorder (designed by L. STIGMARK, Ph. D.). The blood was returned to the animal by intravenous drop infusion. The output of the interval recorder as well as the output of the electromanometer were connected to mirror galvanometers in a modified ECG-machine in which the film was running at a speed of 10 mm/30 seconds.

The EEG was recorded with a two-channel EEG-machine (Grass) in two "bipolar" leads over the cruciate and middle suprasylvian gyri by means of four small gilded silver balls mounted extradurally in the acrylate at the border of the craniotomy. In some experiments needles for stimulation of the reticular activating system of the mesencephalon were mounted stereotaxically in the acrylate material.

Stimulation of the cervical sympathetic nerves was carried out either with indwelling silver electrodes or directly on the dissected nerve (usually divided from the beginning) in a heated paraffin pool. A square wave stimulator (Grass) was used connected to a transformer at the output. The vagus nerve as well as the superior cardiac nerve were cut bilaterally in most experiments and removed for about 3 cm under the place where the sympathetic trunk was stimulated.

In three experiments a rightsided, and in one, a bilateral thoracotomy was made. The thoracic sympathetic chain was cut and dissected free after section of the first 3 thoracic communicant rami according to the technique of BEICKERT, GISSELSSON and LÖFSTRÖM (1956). In these experiments the superior as well as the inferior cardiac nerves were also sectioned.

In 6 experiments section of the sinus nerves was made.

Pupillary dilatation and retraction of the nictitating membrane served as a control of the stimulations.

Results.

1. *Stimulation of the cervical sympathetic nerves.*

Unilateral as well as bilateral stimulation was carried out. Unilateral stimulation was never observed to give bilateral pupillary dilatation. The method of recording cortical blood flow from a midline vein does not permit any conclusions as to side-differences or local differences in cerebral vasomotor effects.

In general, it was found that retraction of the nictitating membrane had the lowest threshold, followed closely by pupillary dilatation. Both these responses were prompt and complete on supramaximal stimulation. Cortical vasoconstriction or other changes in the cortical blood flow were, on the other hand, slower in development, reaching their peak about 15—30 seconds after

the onset of stimulation. With the method used the latency of the onset of vasoconstriction was found to be very short (2—5 seconds) in sensitive preparations. This is in contrast to what has been found with the cranial window technique and with thermocouple measurements (FORBES and WOLFF 1928, SCHMIDT 1934, 1935, 1950, THOMAS 1935, SCHMIDT and HENDRIX 1938, LUDWIGS and SCHNEIDER 1954).

We have confirmed the findings of SCHMIDT (1935) and LUBSEN (1941) that weak cervical sympathetic stimulation often may give a transient increase of the cerebral blood flow which, however, on stronger stimulation changes into a decrease (Fig. 6). We are inclined to conclude with the authors mentioned that the increase is due to a redistribution of blood from extracranial into intracranial pools since the vasoconstriction is known to be much more pronounced in extracranial vessels. The cortical vasoconstriction obtained on supramaximal (unilateral or bilateral) cervical sympathetic stimulation gave a relative diminution of the flow ranging from 10 to 20 per cent.

Very often changes in the blood pressure were observed during cervical sympathetic stimulation. In preparations with intact sinus nerves a slowly developing fall was obtained which was probably due to the effect described by PALME (1943) and by KEZDI (1954). According to these authors cervical sympathetic stimulation produces vasoconstriction in the sinus region which leads to a central inhibition of the vasoconstrictor tone (*cf.* LANDGREN, NEIL and ZOTTERMAN 1952). When blood pressure effects were obtained, a cerebral vasoconstriction was only said to have taken place when the record clearly demonstrated that the diminution of the cortical blood flow was larger than could be seen with spontaneous changes of blood pressure of the same magnitude (Fig. 1 A, Fig. 6). In order to avoid artifacts due to movements and changes in intra-thoracic pressure Flaxedil was often administered. Although this drug obscured the pupillary reactions, vasoconstrictor responses did not seem to be diminished (Fig. 1 B).

Bilateral stimulation of the sympathetic chains usually did not give more pronounced effects than did unilateral. One example is demonstrated in Fig. 2. In this experiment, in which the sinus nerves had been divided bilaterally there was a small initial increase of the blood pressure. In spite of this a slight diminution of the cortical blood flow occurred which, however, after a further

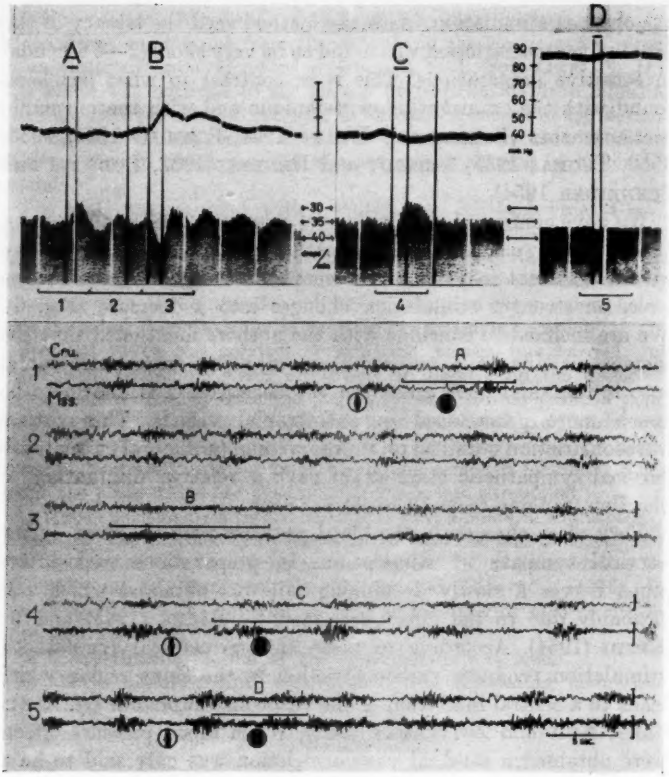

Fig. 1. Cat. Nembutal anaesthesia. Sinus nerves and vagi divided. Adrenalectomized.

Upper diagram: Records of blood pressure (thick line) and cortical blood flow (vertical lines). Each vertical line corresponds to a blood drop from the superior sagittal sinus. Length of vertical lines is proportional to the interval between the blood drops. A line joining upper ends of the vertical lines gives record of cortical blood flow in rostral parts of the brain. Interruptions of record every 30 seconds.

Lower diagram: EEG samples (bipolar leads from the cruciate (Cru.) and the middle suprasylvian (Mss) gyri), corresponding to periods indicated by numbers below upper diagram. Pupillary reactions indicated.

A: Unilateral supramaximal stimulation (20/sec., 1 msec., 1.5 V) of the cut cervical sympathetic nerve. *B:* Pinching the forepaw. Note slight reduction of cortical blood flow with concomitant pupillary reaction upon sympathetic stimulation and increase in flow upon pinching the paw; in the first case accompanied by a small decrease of pressure with unchanged EEG, in the last case by an increase of pressure with a slight arousal reaction in the EEG. *C:* Sympathetic stimulation (20/sec., 1 msec., 1.5 V) after Flaxedil and artificial respiration. *D:* Sympathetic stimulation (increased intensity) after Ergotamine (0.2 mg/kg). There is no change of blood flow but a preserved pupillary dilatation.

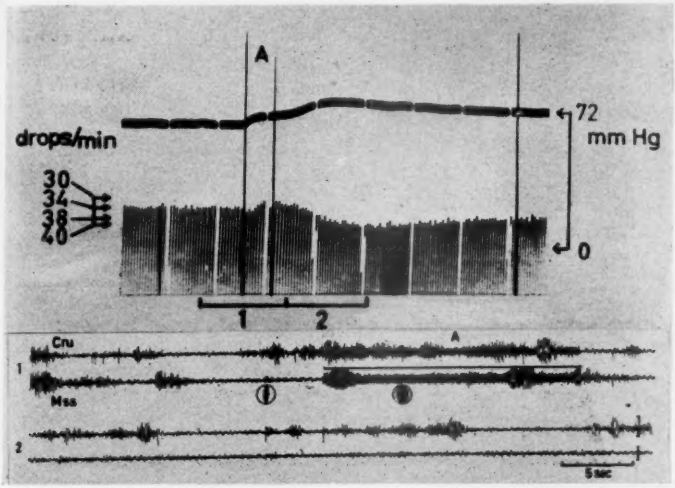

Fig. 2. Cat. *Encephalé isolé*-preparation. Sinus nerves and vagi divided. Records of blood pressure, cortical blood flow and EEG as in Fig. 1.

A: Bilateral supramaximal stimulation (50/sec., 2 msec., 3 V) of the cut cervical sympathetic nerves. Note initial reduction of cortical blood flow in spite of a small increase of blood pressure. There is then a further increase of blood pressure which coincides with an arousal reaction in the EEG (due to spread of the stimulus). During this period the cortical blood flow changes into an increase.

increase of the blood pressure, was changed into a slight increase of flow. The later part of these events were probably provoked by spread of the stimulus and a concomitant late arousal reaction which is seen in the EEG (see below).

On several occasions it was tried to obtain cerebral vasoconstriction, when the cerebral vessels were dilated. Large effects of sympathetic stimulation have previously been reported after administration of CO_2 and histamine (SCHMIDT 1934). In principle, it was confirmed in the present experiments that sympathetic cerebral vasoconstrictor effects are more pronounced when the cerebral vessels are in a dilated state, although with the preparations used blood pressure effects were then also more pronounced.

The cerebral vasoconstriction obtained from cervical sympathetic stimulation could not be elicited after the administration of ergotamine (0.15 mg/kg) (FORBES and COBB 1938) although a pupillary dilatation still could be traced (Fig. 1 C).

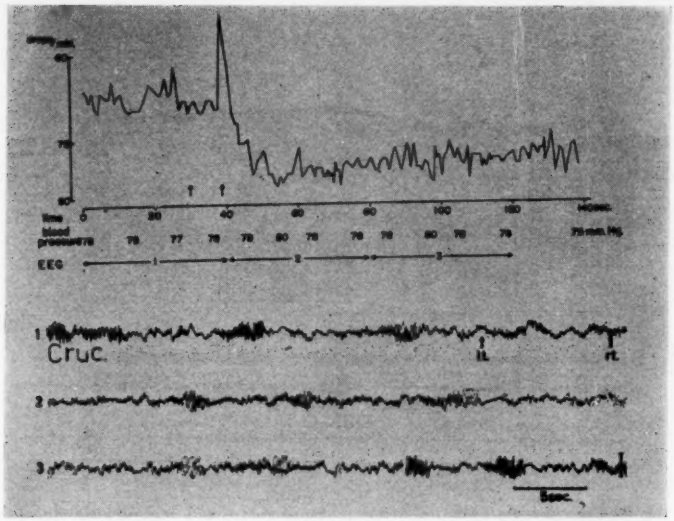

Fig. 3. Cat. Light Nembutal anaesthesia. Vagi divided. Records of cortical blood flow, blood pressure and EEG. The flow record plotted from measurements of interval between drops.

Arrows indicate section of right and left cervical sympathetic nerves. Following the sections there is an increase of the cortical blood flow without any change in the EEG. In this experiment nociceptive stimulation was followed by an immediate arousal reaction in the EEG.

2. *Section of the cervical sympathetic nerves.*

In 3 of the Nembutal preparations acute sections of the cervical sympathetic nerves were carried out in order to study the vasoconstrictor tone. In some instances no increase of flow was obtained after the section, but in others a definite reduction of the cerebral vascular resistance took place which was not accompanied by any significant change of the blood pressure or the EEG (Fig. 3).

3. *Stimulation of the thoracic sympathetic chain.*

These stimulations were carried out unilaterally or bilaterally (1 experiment) in the hope of obtaining a maximal sympathetic vasoconstriction in cerebral vessels including the vertebral artery and its ramifications. With the method used, however, which primarily records blood flow from the rostral and parietal parts of the brain, the cortical vasoconstrictor responses obtained

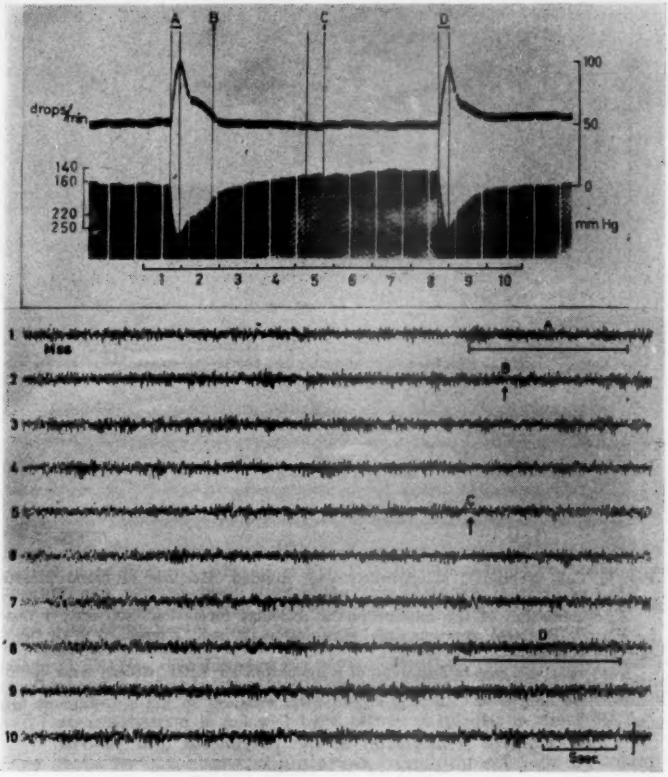

Fig. 4. Cat. Nembutal anaesthesia. Vagi divided. Records of blood pressure, cortical blood flow and EEG as in Fig. 1.

A and D: Stimulation of the mesencephalic reticular formation (250/sec., 1 msec., 1.5 V). Between these stimulations the left (*B*) and right (*C*) cervical sympathetic nerves were sectioned.

Note that the brainstem stimulations give rise to equal blood pressure reactions and very similar (slight) arousal reactions in the EEG. The increase of cortical blood flow is, however, slightly larger in *D* after section of the sympathetic nerves.

were, in fact, smaller than with the cervical stimulations. One reason for this was probably the increase of blood pressure which usually was a steady accompaniment to stimulation of the thoracic sympathetic chain. Such blood pressure effects persisted after the section of the cardiac nerves.

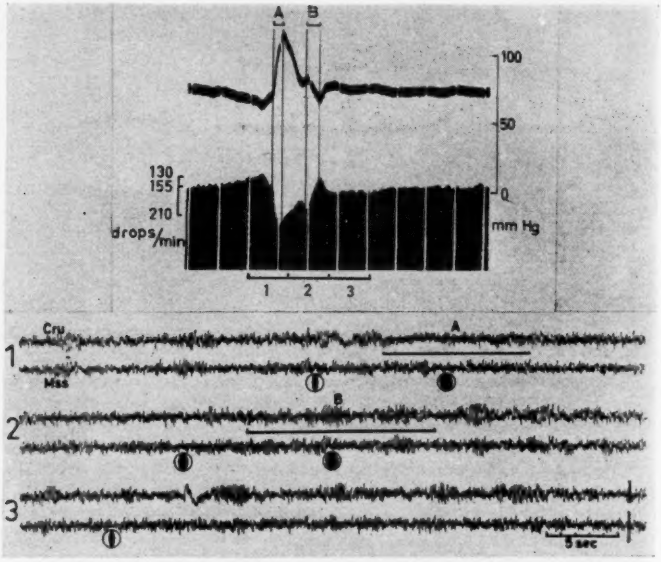

Fig. 5. Cat. Nembutal anaesthesia. Vagi divided. Records of blood pressure, cortical blood flow and EEG as in Fig. 1.

A: Stimulation of the mecencephalic reticular formation (250 sec., 1 msec., 1.5 V). *B:* Bilateral supramaximal stimulation (20/sec., 1 msec., 3 V) of the cut cervical sympathetic nerves.

Note that the re-establishment of a synchronized EEG pattern with spindles after the arousal reaction is enhanced by the sympathetic stimulation.

4. *Control stimulation of the peripheral and central parts of the vagus nerve.*

These stimulations (in Nembutal animals) were always followed by a very prompt fall of blood pressure which, as expected, was more pronounced when the peripheral end was tried. There was a diminution of the cortical blood flow proportional to the fall of blood pressure (Roy and Sherrington 1890).

Stimulation of the central end did not give pupillary effects or any effects upon the EEG.

5. *EEG effects.*

Most of the observations were carried out in preparations which were either non-anaesthetized (*encéphale isolé*) or under light anaesthesia. This permitted a change of the cerebral excita-

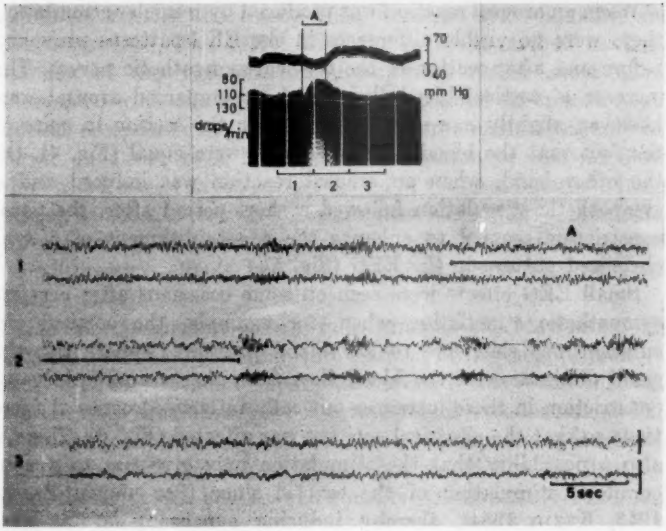

Fig. 6. Cat. Nembutal anaesthesia. Vagi divided. Records of blood pressure, cortical blood flow and EEG as in Fig. 1. White lines indicate increasing blood pressure and cortical blood flow due to intravenous infusion.

A: Bilateral supramaximal stimulation (20/sec., 1 msec., 3 V) of the cut cervical sympathetic nerves.

Following the stimulation there is an initial increase of the cortical blood flow (see text). The flow then decreases due to vasoconstriction. Compare blood pressure reactions and changes of cortical blood flow during the stimulation at A and during the spontaneous fall of pressure later in the record. The sympathetic cerebral vasoconstriction is followed by a period of spindles in the EEG.

tory state by adequate stimulation (pinching etc.) or by means of electrical stimulation of the brain stem reticular formation (MORUZZI and MAGOUN 1949). From the EEG point of view such preparations might be considered as very sensitive with an intact and functioning brain stem.

In most instances in which cervical sympathetic stimulation was carried out in the resting preparation, leading to a definite cerebral vasoconstriction, there were no effects seen in the EEG. It should be stressed that this was also the case when, for example, pinching of a paw gave an arousal reaction (Fig. 1 A). On several occasions, however, it was observed on stronger stimulation of the cervical sympathetic trunk, giving a spread of the stimulus to the neck muscles, that there was an arousal reaction in the EEG which was not specific to the sympathetic stimulation (Fig. 2).

When an arousal reaction was produced by reticular stimulation there were no visible differences in the EEG-patterns provoked before and after section of the cervical sympathetic nerves. The increase of cortical blood flow which accompanied arousal was, however, slightly more pronounced after the section in spite of the fact that the blood pressure effects were equal (Fig. 4). On the other hand, when an arousal reaction was induced and a sympathetic stimulation followed a short period after, the vasoconstriction seemed to enhance the re-establishment of a synchronized pattern in the EEG (Fig. 5).

Small EEG effects were seen on some occasions after cervical sympathetic stimulation when, for example, the cortical circulation was slow due to low blood pressure. Apparently, the small diminution of the blood flow from the sympathetic vasoconstriction in these instances critically influenced cerebral functions so that the electrical activity was affected (Fig. 6). There is also a possibility that the stimulation may give rise to a concomitant stimulation of the carotid sinus (See above: PALME 1943, KEZDI 1954), thereby inducing synchrony in the EEG (BONVALLET, DELL and HIEBEL 1954, NAKAO, BALLIM and GELLHORN 1956).

Discussion.

A discussion has been given elsewhere of the method used here for recording cortical blood flow (INGVAR and SÖDERBERG 1956 a and b, INGVAR 1957).

In the present study of cerebral sympathetic vasoconstrictor effects no measures have been taken to eliminate the extracranial circulation in order to study pure intracranial sympathetic events. This would have required much larger operative procedures (FINESINGER and PUTNAM 1933, BOUCKAERT and JEURDAN 1949, SCHMIDT and HENDRIX 1938) which probably would have jeopardized those sensitive brain stem functions which were a prerequisite for the state to be studied. The size of the internal carotid artery in the cat would also have made any attempt to isolate the intracranial circulation complicated indeed (CHUNGCHAROEN, DE BURGH DALY, NEIL and SCHWEIZER 1952, BATSON 1944).

The vasoconstriction found in the present series as well as the vasodilatation on section of the cervical sympathetic nerves have been of the same magnitude as those found by other authors

(FORBES, FINLEY and NASON 1933, SCHMIDT 1935, SCHMIDT and HENDRIX 1938, FOG 1939). With the sensitive method used the latency of the vasoconstriction appeared rather short (about 5 seconds) while the development of a maximal effect took about 20—30 seconds. In our experience even supramaximal stimulation never gave rise to a long-lasting diminution of cortical blood flow which would have justified the use of the term "spasm". Long-lasting decrease of flow after a stimulation was always accompanied by a decrease of blood pressure. The rôle of the sinus for such blood pressure effects has been discussed above (PALME 1943, KEZDI 1954).

In the experiments in which continuous observations of the EEG and cortical blood flow were made at the moment of bilateral section of the cervical sympathetic nerves, it was found that this section only sometimes gave rise to cerebral vasodilatation. This is probably explained by the fact that the peripheral sympathetic tone varies with the degree of wakefulness (or with the depth of anaesthesia in the present experiments) (BONVALLET, DELL and HIEBEL 1954).

The experiments related above in which the arousal reaction was studied may, to some extent, elucidate the interplay between different cerebral vasomotor mechanisms during changes in cerebral excitability. In arousal the most conspicuous phenomenon from the cerebral circulatory point of view is the increase in cortical circulation. When changes of blood pressure are excluded, this increase is caused by a reduction of the vascular resistance which is probably mainly due to a change of cerebral metabolism induced by the arousing stimulation (INGVAR 1955, INGVAR and SÖDERBERG 1956, 1957, INGVAR 1957). Although arousal may also involve an increase of peripheral sympathetic tone (BONVALLET, DELL and HIEBEL 1954) the ensuing cortical vasoconstriction which is not very powerful would seem to be completely dominated by the metabolic vasodilatation. The rôle of cerebral parasympathetic vasomotor activity in arousal is at present being analyzed.

From the present experiments it seems justified to conclude that cerebral sympathetic vasomotor changes normally do not influence the cerebral electrical activity. This conclusion is strongly supported by the observation of MEYER, FANG and DENNY-BROWN (1954) who did not find any significant changes in cerebral oxygen "availability" after cervical sympathetic

stimulation or section. Furthermore, the sympathetic vasomotor innervation seems to play a subordinate rôle for the cerebral circulatory adjustments during general changes in the EEG such as those which accompany changes in degree of wakefulness. If, however, the cerebral blood flow is already barely sufficient, due to low blood pressure or due to increased metabolic demands during activation (arousal), a sympathetic vasoconstriction may then change the EEG. Such critical conditions are difficult to reproduce experimentally for a systematic study but they would seem to merit a further analysis, especially from the clinical point of view, since it may be expected that, for example, epileptogenic foci would be more susceptible than normal cortex to sympathetic vasoconstriction.

Summary.

1. The influence of electrical stimulation of the cervical sympathetic nerves and the thoracic sympathetic chain as well as the central and peripheral parts of the cut vagi upon the cortical blood flow and the EEG has been investigated in unanesthetized (encéphale isolé) and lightly anaesthetized cats, sensitive to arousing stimuli.

2. It has been confirmed that stimulation of the cervical sympathetic nerves gives a cerebral vasoconstriction which, according to the method used, may reduce the blood flow by 10—20 %. In lightly anaesthetized preparations section of the same nerves gave a significant reduction of the cerebral vascular resistance (increase of flow of up to 25 %). Stimulation of the thoracic sympathetic chain did not give rise to a more pronounced cortical vasoconstriction than did cervical stimulation.

3. In preparations with intact sinus nerves cervical sympathetic stimulation was accompanied by a slight decrease of blood pressure (PALME 1943). Blood pressure alterations were less pronounced after section of the sinus nerves. Vagal stimulation gave a decrease of the blood pressure and a proportional decrease of the cortical blood flow.

4. In the large majority of cases, maximal sympathetic vasoconstriction was not followed by any change of the EEG. Some observations indicate, however, that when the cerebral blood flow was critically low, a small further decrease due to sympathetic vasoconstriction influenced the EEG.

5. The sympathetic vasomotor innervation of cerebral vessels probably plays a subordinate rôle for the circulatory adjustments in the brain during changes in its state of excitability.

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Heat Regulation and Metabolism in some Tropical Mammals and Birds.

By

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Heat balance in mammals and birds can be maintained down to the critical temperature simply by increasing the insulation, but below this the heat production must be increased. This increase is usually more or less linearly proportional to the rectal to air temperature gradient, in agreement with Newton's law of cooling (cp. SCHOLANDER, HOCK, WALTERS, JOHNSON and IRVING 1950). Much knowledge has been derived from the study of arctic and temperate-zone animals, but only little is known of the temperature reactions in tropical animals. This category is of special interest, because here belongs naked man.

In the present paper the reactions of some tropical mammals and birds to lowered air temperatures will be described. The experiments were carried out at the Canal Zone Biological Area on Barro Colorado Island.

Material and Method.

The animals were caught in the Canal Zone and kept outdoors in wire cages on Barro Colorado Island where the temperature rarely varied more than a few degrees above and below 25° C. They are listed below with their distribution.

Lesser anteater (*Tamandua tetradactyla* L.). Argentine and Paraguay to Mexico (only registered resting metabolic rate).

Opossum (*Didelphis marsupialis* L.). Paraguay and northern Argentine to Panama.

Three-toed sloth (*Bradypus griseus griseus* Gray). Central America south to the Canal Zone.

Mouse (probably *Oryzomys talamancea* Allen). Central America.
Squirrel (probably *Sciurus variegatoides* Goldman). Central America.
Armadillo (*Dasypus novemcinctus* L.). Southern Brazil and Argentine to Texas and Louisiana.
Frigate-bird (*Fregata magnificens* Mathews). Tropical coasts of America (Atlantic and Pacific oceans) and West Africa.
Turkey vulture (*Cathartes aura* L.). North America to 50° N latitude, Central America and West Indies, and South America.
Black vulture (*Coragyps atratus* Bechst.). Middle and northern South America to Kansas and Maryland.

Heat production was determined by indirect calorimetry, using an open circuit system as described by SCHOLANDER et al. (1950). The animals were kept at each temperature for two to three hours, allowing half an hour of equilibration for the small animals and one hour for the big. The flow of air through the system was adjusted to give a carbon dioxide percentage between 0.50 and 0.75 per cent. The gas was analysed with a $^1/_2$ cc gas analyzer (SCHOLANDER 1947).

For graphic representation of the results, the resting metabolic rate was taken as a base line and given the numeric value of 100. Other values are given in per cent of this, and the whole sequence of data is compared with a linear curve, drawn according to Newton's law of cooling.

Results and Discussion.

Metabolic response in mammals. Of the five species of mammals investigated, two are seemingly good thermoregulators, namely the mouse and the squirrel. It will be seen from fig. 1 D and E, that these have a critical temperature of about 27° C. The spread of data is small and their response to cold very closely follows Newton's law of cooling. It is likely that their accurate response is facilitated by their small heat capacity.

The three other mammals, namely the armadillo, opossum and three-toed sloth are poor thermoregulators which lost considerably in body temperature during the cold stress (cp. fig. 1 A—C). A critical temperature cannot be sharply defined, but seems to be above 25° C. The poor temperature regulation in these animals is well known (WISLOCKI and ENDERS 1935, SCHOLANDER and KROG 1957). It is of interest, however, that some species with poor thermoregulation are capable of producing a good metabolic response to lowering of the temperature, such as several of the monotremes and marsupials investigated by MARTIN (1903) and the two-toed sloth (*Choloepus hoffmanni*) (SCHOLANDER et al. 1950).

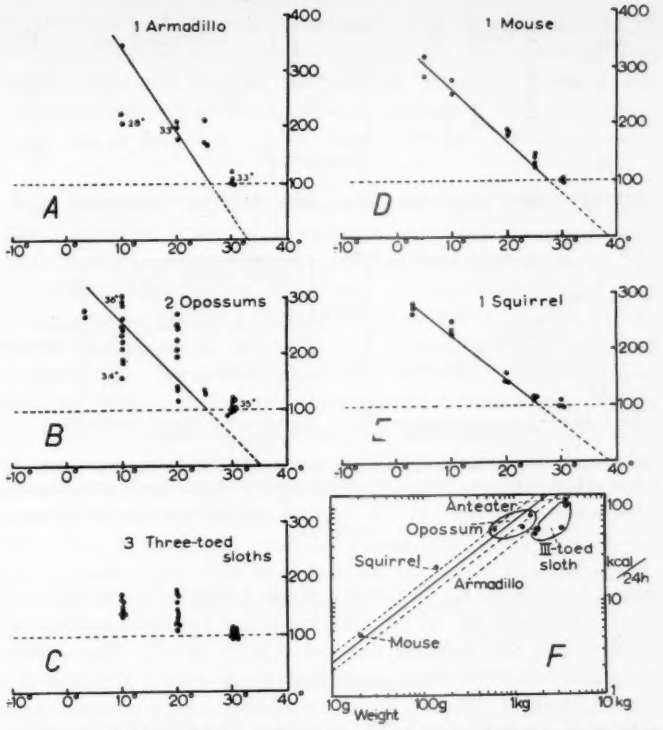

Fig. 1. A—E. Metabolic heat regulation in tropical mammals. *F.* Basal metabolic rate in relation to body size. Full drawn line is the standard curve drawn according to the equation kcal./day $= 70 \times \text{kg}^{\frac{3}{4}}$. Dotted parallel lines are 20 per cent deviations.

Metabolic response in birds. Two species of vultures and one species of frigate-bird were investigated. All three species are soaring birds which often fly at high altitude in relatively cold air. The body temperature (T_b) and critical temperature (T_c) in the vultures are respectively 41° and 24° C; in the frigate-bird they are 38° and 20° C. These relatively low critical temperatures might be an adaptation to the high altitude soaring habit of these birds.

The critical gradient (T_b—T_c) is about the same in all three species. So is also the resting heat production (fig. 2 D) and one might therefore infer that the overall body insulation is about the

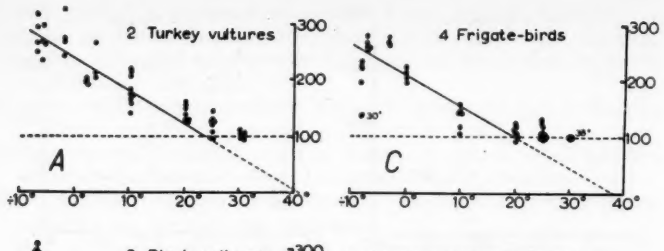

Fig. 2. A—C. Metabolic heat regulation in tropical birds. *D.* Basal metabolic rate in relation to body size. Full drawn line represents the standard curve according to the equation kcal./day $= 89 \times kg^{\frac{3}{4}}$. Dotted parallel lines represent 20 per cent deviations.

same in all of them. The vultures are nevertheless considerably more cold hardy. At —7° C the frigate-bird lost body temperature rapidly, but the vultures did not (fig. 2 A—C). The vultures have also extended their range into much colder latitudes than the frigate-bird. These differences must lie in a greater ability to produce heat during cold in the vultures, which is also indicated from fig. 2 A—C.

Resting metabolic rate. The basal heat production might be considered a potential factor in climatic adaption, *i. e.* overheating in a tropical animal would be counteracted by a low basal metabolic rate (cp. SCHOLANDER et al. 1950). The resting rates have been calculated, assuming RQ = 0.8 and a caloric equivalent of one liter of oxygen = 4.8 kilocalories. In fig. 1 F the data of the mammals are plotted in relation to the standard mouse to elephant curve (BENEDICT 1938, BRODY 1945). Fig. 2 D shows the rates of the birds in relation to the curve for birds given by BRODY (1945).

The mouse, opossum and squirrel agree pretty well with the standard curve and consequently do not show any metabolic adaptation to tropical climate. The Xenarthra group, namely the sloth, armadillo and anteater, fall below the standard curve (cp. OZORIO

DE ALMEIDA and BRANCA A. FIALHO 1924, SCHOLANDER et al. 1950) and likewise the three birds. However, the generally large spread in bird data and the oddity of the Xenarthra group do not invite conclusions as to the climatic significance of these deviations.

Summary.

Five species of tropical mammals and three species of high soaring birds in Panama have been subjected to various air temperatures and their heat production was determined by the carbon dioxide production. The mouse and the squirrel were good thermoregulators with a critical temperature of about 27° C, whereas the opossum, armadillo and three-toed sloth were bad regulators. The birds have relatively low critical temperatures, about 24° (vultures) and 20° C (frigate-bird), which may reflect their habit of soaring in cool air at high altitudes. At sub-critical temperatures the metabolism in both mammals and birds increased with decreasing temperatures, more or less closely approaching Newton's law of cooling.

The resting metabolic rates in mouse, squirrel and opossum were in accordance with the standard mouse to elephant curve. Mammals of the suborder Xenarthra (anteater, sloth, armadillo) and the three bird species had a relatively low metabolic rate.

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Species Differences of Clotting Factors in Ox, Dog, Horse, and Man.

Thrombin and Fibrinogen.

By

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The species specificity of fibrinogen is definitely established, whereas thrombin has been regarded as a distinct substance irrespective of its origin within the mammals.

Studies on the species specificity of fibrinogen have followed different lines, *i. e.* salting out experiments (HUISKAMP 1905, DAVIDE 1925, WÖHLISCH 1929); antigen properties of different fibrinogens (HEKTOEN and WELKER 1927); differences in the clotting times of different fibrinogens or plasmas with the same thrombin (SEEGERS and SMITH 1942, FANTL and EBBELS 1953, BURSTEIN and GUINAND 1954); differences in the optimal pH for the action of thrombin, and in the isoelectric point under similar conditions (ASTRUP and DARLING 1942); differences in the solubility of fibrinogens in ethanol-water mixtures, and in rigidity and opacity of the fibrin clots (MORRISON, SHULMANN and BLATT 1951); and finally differences in the N-terminal residues among fibrinogens (LORAND and MIDDLEBROOK 1953). These investigations convincingly demonstrate the species specificity of fibrinogen.

Where thrombin is concerned, only two observations have been reported which indicate a species specificity. BRYCE (1954 a) found that while sheep thrombin had the same activity in sheep and bovine plasma, bovine thrombin was more active than sheep thrombin in bovine plasma. The other observation was made by

INGRAM (1955), who observed that human and bovine thrombin reacted somewhat differently with human plasma. A statement of BRYCE in another paper (1954 b) is probably an adequate expression of the current view on this problem: "It seems reasonable to treat thrombin as a single substance for purposes of reaction in simple solution of purified fibrinogen. In plasma, however, there is the possibility of specificity in the inactivating mechanisms which cannot be ignored until it can be shown not to exist."

Besides a confirmation of the species specificity of fibrinogen, this paper will present evidence that thrombin is also species specific, and to such a degree that it is necessary to take the specificity into account in thrombin time studies in different species of mammals.

Materials and Methods.

Thrombin. Thrombin was made from citrated plasma of ox, dog, horse, and man. The plasma was diluted 15 times with cold distilled water and the pH was adjusted to 5.3 with one per cent acetic acid. After standing for two hours at 4° C, the precipitate was collected by centrifugation and dissolved in veronal buffer. Homologous thromboplastin and calcium chloride to a concentration of 2.5 mM were added, and the mixture was incubated in a waterbath at 37° C for one hour to allow for complete conversion of prothrombin to thrombin. The fibrin was removed and the thrombin solution was dialyzed against saline at neutral pH in the cold to remove calcium and buffer. The thrombin solution was then centrifuged for one hour at 40,000 rpm to remove the thromboplastin, and stored at — 20° C.

Fibrinogen. Fibrinogen from ox, dog, and man was prepared according to the method of KEKWICK and MACKAY (1954). This ether precipitation method, however, did not work satisfactorily with horse plasma, and fibrinogen from this species was prepared by isoelectric precipitation at pH 6.2 (ASTRUP and DARLING 1942). The preparations were washed twice and then dissolved in saline. In the key experiments, the fibrinogen solutions were used immediately after preparation. Optimal concentrations of fibrinogen solution for the thrombin test were worked out for each species preparation.

Saline brain thromboplastin was prepared according to the method of Owren (1949) and stored in aliquots at — 20° C.

Citrated and oxalated plasmas were obtained from blood drawn into one tenth volume of sodium citrate dihydrate 3.1 per cent or potassium oxalate monohydrate 2.5 per cent. The plasmas were separated after centrifugation in the cold for 30 minutes at 2,500 rpm. Only fresh plasmas were used.

Adsorbed, dialyzed plasma was prepared from oxalated plasma by adsorption with 100 mg per ml of barium sulphate (BAKER), followed by dialysis in the cold against saline for 24 hours.

Veronal buffer. A modified veronal buffer (OWREN 1947) was made by mixing sodium diethyl barbiturate 11.75 g; sodium chloride 14.67 g; 0.1 N HCl, 430 ml; and distilled water to 2,000 ml (pH 7.35, ionic strength 0.154).

Dialysing casings. Cellophane casings manufactured by The Visking Corporation, Chicago, were used.

Assay of fibrinogen. One ml of citrated plasma was mixed with 15 ml of veronal buffer in a centrifuge tube, and one ml of thrombin solution containing 30 N. I. H. units was added. The mixture was continuously stirred with a glass rod until all fibrin formed had adhered to the rod. The rod was left in the mixture for one hour to allow further formation of fibrin, but as a rule no additional fibrin formed after a few minutes, even after addition of more thrombin. The fibrin clot was wound on the glass rod and washed once in saline and twice in distilled water. Each washing lasted for half an hour. All procedures were carried out at room temperature. The nitrogen content of the washed fibrin was assayed with the KJELDAHL method. In the calculation of the fibrinogen concentration, 6.25 was used as the nitrogen factor.

pH-measurements were done electrometrically with a Radiometer pH-meter (Copenhagen). Accuracy 0.05 pH units.

Measurement of specific conductivity was performed with a Philips conductivity-measuring bridge GM 4249/01.

Experiments.

A. *The fibrinogen concentration in ox, horse, and dog plasma.*

The plasmas used for fibrinogen assay were obtained from non-pregnant, healthy animals. Citrated plasma was used, and was refrigerated if not used immediately. The figures presented are corrected for the anticoagulant solution used, according to the hematocrit, but not for the fibrinopeptide split off during fibrin formation, which amounts to about 3 per cent of the fibrinogen (LORAND 1952). As is seen in table 1, ox plasma contains about twice as much fibrinogen as horse and dog plasma, which have about the same concentration as human plasma (ALBRITTON 1953). However, there are great individual variations within each species.

B. *Experiments with adsorbed, dialyzed plasmas.*

In the following experiments the conditions necessary for designing a valid experiment on the species specificity of thrombin and fibrinogen were investigated.

Table 1.

The fibrinogen concentration in plasmas from ox, horse, and dog as measured with the Kjeldahl method.

Species	Number of animals	Fibrinogen g per 100 ml	
		Mean	Range
Ox	10	0.56	0.45—0.75
Horse	10	0.30	0.23—0.38
Dog	10	0.28	0.22—0.38

The fibrinogen concentration in a mixture of ten normal human plasmas was estimated to 0.31 g per 100 ml with this method.

Oxalated plasmas from ox, horse, dog, and man were adsorbed with 100 mg per ml of barium sulphate; dialyzed in the same container against 0.9 per cent saline; and handled in the same way throughout the experiment. After dialysis, the specific conductivity was found to be nearly identical in all plasmas, indicating a close similarity in the ionic strength.

1. *The optimal test system for evaluating thrombin activity in different plasmas.*

Since the concentration of fibrinogen differed in the plasmas, it was necessary to test the optimal plasma dilution for the thrombin time test. As is seen from table 2, the following mixtures gave the shortest clotting times for the thrombin concentrations used in these experiments. Human 0.6 ml plasma + 0.2 ml thrombin; bovine 0.1 ml plasma + 0.5 ml saline + 0.2 ml thrombin; horse 0.3 ml plasma + 0.3 ml saline + 0.2 ml thrombin; dog 0.3 ml plasma + 0.3 ml saline + 0.2 ml thrombin. When compared with the concentration of fibrinogen in plasma, it is seen that the optimal plasma dilution is not solely determined by the concentration of fibrinogen. The mixtures mentioned were used in all subsequent experiments with plasma.

2. *The optimal pH for the thrombin-fibrinogen reaction in plasmas of different species.*

Preliminary experiments indicated that the pH-thrombin time curves differed for plasmas from different species, and the optimal pH for the thrombin test, therefore, had to be worked out for each plasma. N/2 HCl and N/2 NaOH were used to adjust pH.

Table 2.

The optimal plasma dilution of different plasmas in the thrombin fibrinogen reaction.

Plasma ml Saline ml	0.1 0.5	0.2 0.4	0.3 0.3	0.4 0.2	0.5 0.1	0.6 0.0
Human plasma	28.5	25.5	24.0	22.1	21.2	20.9
Bovine "	24.1	25.5	28.0	31.0	36.1	39.2
Horse "	32.5	30.4	30.3	31.1	32.0	34.4
Dog "	17.2	16.9	16.5	17.1	17.3	17.5

To the mixtures indicated in the table, 0.2 ml bovine thrombin solution was added, and the clotting times in seconds were measured.

Preliminary experiments also showed that the pH-thrombin time curve was changed by dialysis, with the pH optimum for plasmas being higher after dialysis. This may be due mainly to an increase in the pH of the nondialyzed plasma in the interval between the measurement of pH and the thrombin assay. Consequently, the the thrombin time was measured at a higher pH than expected. Possibly some change in ionic strength also took place during dialysis, thereby altering the optimal pH.

The results obtained with bovine thrombin in the different plasmas are presented in figure 1. First, it is seen that the optimal pH values were 7.35, 7.20, 7.30 and 6.95 for human, ox, dog, and horse respectively. Secondly, the difference in reactivity of the different plasmas is obvious. These differences increased with increasing pH, as indicated by the divergence of the right parts of the curves. Although the differences are small at optimal pH, they are still definite.

3. *The optimal pH for the effect of different thrombins in the same plasma.*

These experiments were designed to find out whether the optimal pH varied with the type of thrombin used. According to the experiments of ASTRUP and DARLING (1942), this would not be very probable. The results with bovine plasma are shown in figure 2. The optimal pH values for different thrombins tested on the same plasma were much the same. Horse thrombin required a slightly lower pH for its maximal effect than the other thrombins

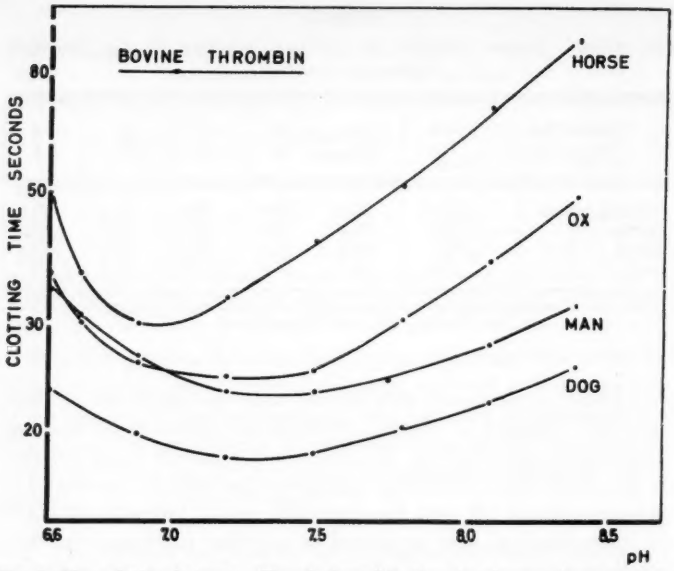

Fig. 1. The relation between pH and thrombin time in plasmas from various species.

tested. However, it is reasonable to conclude that the pH optimum for the thrombin-fibrinogen reaction is largely determined by the type of plasma, and not by the thrombin.

4. *Thrombin dilution curves with plasmas of different species.*

Experiments were carried out to see whether it was justifiable to use the same standard curve in calculating the thrombin concentration in per cent for all plasmas and thrombins used in this study. Thrombin dilution curves, therefore, were made for the four different thrombins on the same plasma, and for the same thrombin on the four different plasmas. The result showed that the curves for ox, dog, and man were closely parallel. The results obtained by reading the same clotting time from any one of the curves did not deviate more than $\pm$ 2 per cent within the range of clotting times used. Horse plasma, however, showed a more gradual curve, and the results with horse plasma had to be read from this curve.

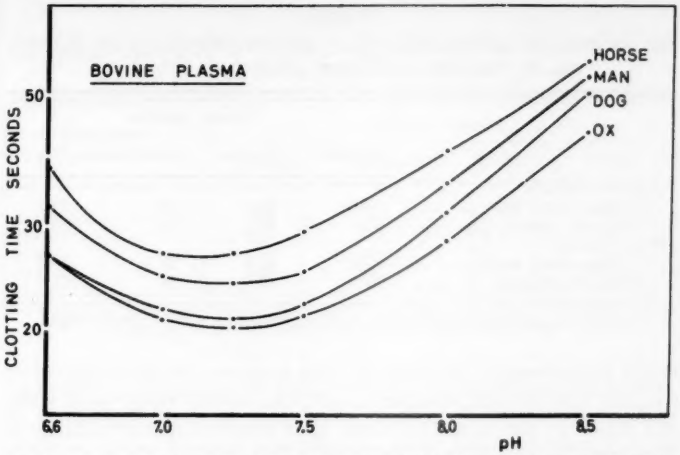

Fig 2. The relation between pH and thrombin time of various thrombins in the same plasma.

5. *Does calcium eliminate the observed differences in the reactivity of various plasmas to thrombin?*

This question is of interest for the evaluation of comparative studies on prothrombin concentration or conversion, since calcium is present in the mixtures and the calculations are based on the amount of thrombin evolved. In the experiment reported in table 3, the thrombin activity was estimated in the different plasmas before and after addition of optimal amounts of calcium. The plasmas were tested at the pH resulting after dialysis, *i. e.* 7.7, and bovine thrombin was used. When the figures in the upper line of table 3 are compared with the figures for bovine thrombin in table 4, it is evident that the differences in reactivity to thrombin among the plasmas were greater at pH 7.7 than at their optimal pH. This might also be deduced from the pH curves in figure 1. If, however, the figures in the lower line of table 3, *i. e.* after addition of calcium, are compared with the figures in table 4, the pattern is found to be similar. The plasmas used in these two experiments were not the same, and this may account for the quantitative differences. The figures in table 3 thus show that the differences in reactivity were maintained to the same degree even when calcium was present. Table 3 with the figures for bovine thrombin in table 4, further indicates that calcium abolishes the influence of pH on thrombin time.

Table 3.

The influence of optimal amounts of calcium chloride on the differences in reactivity of different plasmas to thrombin.

	Plasma species			
	Human	Bovine	Horse	Dog
Without calcium				
Clotting time, secs.	63.6	80.0	120	44.8
Thrombin activity %	100	76	45	150
With calcium				
Clotting time, secs.	24.2	26.8	33.4	18.8
Thrombin activity %	100	89	64	135

The plasma used had a pH of 7.7, and the thrombin was of bovine origin.

Other experiments, designed for this purpose, showed that when calcium was present, changes in pH over a wide range were without effect on the thrombin time, thus confirming the findings of BURSTEIN and GUINAND (1954).

6. *The species specificity of thrombin and fibrinogen as judged from experiments with plasma.*

After the basic conditions had been investigated as reported in the preceding sections, the following cross matching experiment was done. For this experiment a pool of three different freshly drawn plasmas from each species was used. Oxalate was used as the anticoagulant, and the pooled plasmas were prepared as described in the above experiments. The thrombin activity at the optimal pH for each plasma was tested, *i. e.* where the difference in reactivity was least. Thus, the pH was adjusted with N/2 hydrochloric acid according to the findings in section 2. The experiment was conducted as follows: The concentration of thrombin from each species was adjusted to give identical clotting times in human plasma. These thrombin solutions were consecutively tested in the plasmas of the other species. If thrombin were not species specific the clotting times should be the same with all thrombins in each of the different plasmas, although different times would be expected from one plasma to another due to their different reactivities observed above. The results are given in table 4. In presenting the results, the clotting times were converted to per cent thrombin activity by standard curves and the activity in human plasma was taken as 100 per cent. First, the figures in the table illustrate the species

Table 4.

The species specificity of thrombin and fibrinogen as revealed by the activity of different thrombins in different plasmas.

Thrombin species	Plasma species			
	Human	Bovine	Horse	Dog
Human	100	74	65	150
Bovine	100	93	64	125
Horse	100	85	76	115
Dog	100	95	100	220

The figures in the table refer to per cent thrombin activity as converted from clotting times by standard thrombin dilution curves.

specificity of fibrinogen, dog plasma being the most reactive followed by human, bovine, and horse plasma. With dog thrombin, however, horse plasma was slightly more reactive than bovine plasma, indicating thrombin specificity. The specificity of thrombin is best seen when the table is read vertically. Although the effect of the thrombins was the same in human plasma, a great variation was found when the same thrombins were tested in bovine, horse and dog plasma. The following general features might be concluded: Dog thrombin was more active than was the homologous thrombin in every plasma except human plasma. With this exception the effect of each thrombin was greatest in its homologous plasma. This was especially seen in the canine species. A dog thrombin estimated to be 1 T. U. with human plasma would amount to 2.20 T. U. as estimated with its own plasma as the substrate.

B. *Experiments with purified fibrinogen.*

The same preliminary experiments as described in paragraphs 1—4 were performed in order to establish the conditions for a cross matching experiment similar to that reported in table 4. The optimal pH-range for the thrombin fibrinogen reaction with purified fibrinogen was found to be wide for all fibrinogens. The fibrinogens were, therefore, dissolved in veronal buffer at pH 7.35, and used in optimal concentration. As to the thrombin dilution curves, the horse fibrinogen was found to have a slightly more gradual curve than the other fibrinogens, which were nearly parallel. This implied that the results with horse fibrinogen had to be read from the horse fibrinogen curve. The experiment was

Table 5.

The species specificity of thrombin and fibrinogen as revealed by the activity of different thrombins on different fibrinogens.

Thrombin species	Fibrinogen species			
	Human	Bovine	Horse	Dog
Human	100	80	79	240
Bovine	100	90	84	215
Horse	100	79	88	205
Dog	100	105	115	410

The figures on the table refer to per cent thrombin activity as converted from clotting times by standard thrombin dilution curves.

carried out in the same way as the similar experiment with plasma, and the results are shown in table 5. When the results are compared with those in table 4, the same general pattern is found. Concerning the specificity of fibrinogen, it is seen that purified fibrinogen from horse and dog showed a proportionally higher reactivity than when tested in its original plasma. As to thrombin specificity, the greater reactivity of dog thrombin with fibrinogens other than human is again seen, and likewise that the thrombins preferred the homologous fibrinogen. In the dog this was still more pronounced with purified fibrinogen than with plasma. A dog thrombin estimated to be 1 T. U. with human fibrinogen would amount to 4 T. U. as assayed with dog fibrinogen. Despite some minor deviations, the results reported in table 4 and 5 generally correspond. Besides confirming the specificity of fibrinogen, and at the same time giving an impression of its quantitative aspects, the results indicate a definite species specificity of thrombin. Since the results were grossly the same with fibrinogen as with plasma, it might be concluded that plasma factors have little, if any, influence on the species specificity of thrombin.

Discussion.

Only one reference has been found concerning the quantitative assay of fibrinogen in ox, horse, and dog plasma (ALBRITTON 1953). The value obtained for dog in this study deviates markedly from that of the above report, being only half as much, *i. e.* 0.28 g per 100 ml plasma. The reason for this discrepancy is obscure. No significant difference was found between breeds (three breeds

were examined). The values of ox and horse are also somewhat lower than those earlier reported, but the observation is confirmed that ox plasma contains about twice as much fibrinogen as horse plasma.

Some comments on the preparation of the plasmas used in this comparative study are needed. To obtain a valid comparison of the plasmas from different species, the unspecific factors were considered, *i. e.* temperature, ionic strength, pH, anticoagulant concentration, fibrinogen concentration, and the other colloids in plasma. The adsorption served two purposes: (1) Removal of adsorbable clotting factors to exclude influence from the plasma's own clotting system. (2) Removal of adsorbable anticoagulants such as heparin. If plasma really contains significant amounts of heparin as found by NILSSON and WENCKERT (1954), this would invalidate experiments on species specificity of thrombin, since the heparin-cofactor complex also shows definite species specificity (HJORT and STORMORKEN 1957). Furthermore, the simultaneous dialysis of the plasmas against the same dialysis fluid likewise served two purposes: (1) A proper control of pH was made possible. (2) A uniform ionic strength was obtained.

The pH-optima for thrombin activity were estimated after adjustment of pH with N/2 HCl and N/2 NaOH. By doing this, the ionic strength changes somewhat, but not significantly enough to be of importance (ASTRUP 1944). As seen from figure 1, the pH-thrombin time curve varied with the plasma species, and the sensitivity to changes in pH increased in the order dog, man, ox, and horse. This conforms with the findings of BURSTEIN and GUINAND (1954), but in contrast to their findings, a fairly definite pH optimum was found for each plasma. Concerning the purified fibrinogens, the pH optima were less distinct than in plasma. The thrombin time was nearly the same between 7.05 and 7.8, but the lower limit of the optimum varied, being 6.8, 6.9, 7.0, and 7.05 for horse, ox, human, and dog fibrinogen respectively. ASTRUP and DARLING found lower and more definite optima, at least for horse fibrinogen, which might be explained by their use of phosphate buffers at constant ionic strength.

As can be seen from table 4, the general pattern of reactivity of the plasmas, although varying with the thrombin species, was decreasing in the order dog, man, ox and horse. These findings conform with those of BURSTEIN and GUINAND for ox and horse plasmas. SEEGERS and SMITH (1942) found that dog plasma was

less than half as reactive as was human plasma, which is contrary to our findings. Their results with ox plasma versus human plasma are in accord with the present findings.

At least three factors might be responsible for the differences in reactivity among the plasmas: antithrombin, the fibrinogen conversion accelerator (RATNOFF 1954), and the fibrinogen itself. The accelerator of the thrombin fibrinogen reaction of WARE, FAHEY and SEEGERS (1948), platelet factor 2, has not been considered here, because the plasmas were nearly free of platelets. The work of LYTTLETON (1954) makes it improbable that antithrombin should be the cause, since he found antithrombin to be without effect on clotting times shorter than 100 seconds, and the clotting times in this study were generally between 15 and 60 seconds. Experiments were carried out to clarify the influence of the fibrinogen conversion accelerator. The results, which are reported elsewhere (STORMORKEN 1957), indicated that the existence of this factor is doubtful. Of factors known to influence the thrombin fibrinogen reaction, therefore, only the fibrinogen itself was left for consideration as the cause of the difference in reactivity among the plasmas. This was corroborated by the experiments with purified fibrinogen. Except for dog fibrinogen, the results with plasma and purified fibrinogen corresponded fairly well. The explanation for the increase in reactivity of dog and horse fibrinogen on purification is obscure. Possibly some alteration had taken place during preparation. Nevertheless, it may be concluded that the difference in reactivity among the plasmas depends on the chemical nature of fibrinogen.

The investigation of the species specificity of thrombin was the main object of this work, and the figures in the columns of tables 4 and 5 clearly demonstrate that such a specificity exists. As shown by INGRAM (1955), the accuracy of the thrombin-time test is high. He found that the average coefficient of variation, *i. e.* the standard deviation expressed as a percentage of the mean, was about 3 per cent for duplicate readings. Using this value, we can conclude from the thrombin dilution curves that differences above 6 per cent are statistically significant at the 95 per cent level in tables 4 and 5. Thus, there is little doubt that the differences found are significant. As can be seen from tables 4 and 5, the specificity of fibrinogen is generally more important quantitatively than the thrombin specificity. However, for the dog species the

inverse is found, and experiments with dog thrombin might be quite misleading unless the specificity is considered.

The establishment of thrombin specificity makes the thrombin unit still more relative than generally realized. In the author's opinion the thrombin unit should be used chiefly as a commercial measure of thrombin activity. If the thrombin concentration is of special importance for the evaluation of experimental results, the activity should be given in terms of clotting times under the actual, standardized conditions. Only this would enable subsequent workers both to reevaluate the experiments, and to repeat them under similar conditions.

Summary.

1. The fibrinogen concentration has been estimated to 0.28, 0.30, 0.56 g per 100 ml in dog, horse, and ox plasma respectively.
2. The different reactivities to thrombin of various plasma species have been confirmed. The reactivity decreased in the following order: dog, human, ox, and horse plasma.
3. The difference in reactivity of the plasmas was least at optimal pH, and increased with increasing pH.
4. The addition of optimal amounts of calcium did not significantly alter the proportional differences among the plasmas.
5. When bovine thrombin was used, the quantitative differences in reactivity were as follows: dog 125, human 100, ox 93, and horse 65 per cent.
6. Purified fibrinogens from the same species showed the same reactivity pattern as plasmas, but dog fibrinogen showed a proportionally greater activity than dog plasma.
7. Thrombin was found to be species specific. Except in human plasma and in human fibrinogen, dog thrombin was more active than the homologous thrombins. With this exception, the thrombins preferred the homologous plasma or fibrinogen.
8. The thrombin specificity was especially pronounced with dog thrombin. A dog thrombin estimated to be 1 T. U. in human plasma or fibrinogen amounted to 2.2 and 4.1 T. U. as estimated on the corresponding dog materials.
9. On the basis of these findings the author recommends that the use of thrombin units be replaced by clotting times under standardized conditions in scientific work.

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The Respiratory Gaseous Exchange After a Short Burst of Exercise.

By

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The present investigation was undertaken in order to compare the respiratory gaseous exchange of well-trained and untrained individuals after a short sprint.

Thirteen healthy male students between 19 and 23 years of age served as experimental subjects. Some of the students were well trained in athletics, while some were not at all active in sports. Further information about the physical characteristics and participation in sports of the students is given in Table I.

Method.

The experiments were done in a field house with a race track, which was regularly prepared, and conditions therefore uniform throughout the experiments. On this track the subjects ran a distance of 70 yards, preferably using 9 to 10 seconds. The actual time used was measured with a stopwatch. A specially constructed spirometer (ERIKSON, SCHOLANDER and IRVING 1951), which was a modification of the "layering spirometer" (SCHOLANDER 1937), was placed a few yards from the finish line. With this spirometer the respiratory gaseous exchange (*i.e.* carbon dioxide output, oxygen uptake and pulmonary ventilation) was determined during rest and during recovery after the exercise. The subject was connected with the spirometer by a mouthpiece attached to a low resistance breathing valve, and his nose was closed by means of a noseclip. The inner diameter of the mouthpiece was 36 mm, and the i.d. of the tubing and spirometer connections 50 mm. The resistance of the breathing valve was approximately 1 mm of water at a flow rate of 50 l/min increasing to about 2 mm at 100 l/min.

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Table I.

Subject	Age in years	Weight kg	Height cm			Speciality	V.C. l. ATPS
A	20	68	175	Trained	Good form	Mid-Atl. Sts. 1 mile champ. 1/2 & 1 mile	4.6
L	20	64	174	Trained	Good form	Cross country 1 & 2 miles	4.8
M	21	74	183	Trained	Good form	1/4 & 1/2 mile	5.2
G	20	81	191	Trained	Not in good form	1/2 mile	5.6
I	19	66	178	Trained	Not in good form	100 & 220 yards	4.3
D	18	60	173	Trained	Not in good form	Cross Country 1/2 & 1 mile	3.9
B	18	73	185	Untrained	Does not take part in sports		5.4
C	20	73	180	Untrained	Does not take part in sports		—
E	22	70	188	Untrained	Last man in cross country previous year		5.4
F	19	82	173	Untrained	Does not take part in sports		3.8
H	23	66	180	Untrained	Does not take part in sports		5.3
J	18	68	185	Untrained	Plays some tennis		4.8
K	21	70	188	Untrained	Base-ball manager		5.1

The total resistance to breathing (including valve, tubing and spirometer) was about 2 mm at a flow rate of 50 l/min and about 5 mm at 100 l/min. The spirometer was filled with atmospheric air and the carbon dioxide output, oxygen uptake and ventilation were determined during 2 minute periods.

The CO_2 output was determined with an accuracy of ± 3 %. The accuracy of the O_2 determination depends on a constant expiratory level, and is furthermore as in all spirometers of this type, very sensitive to leaks.

The pulmonary ventilation was determined with an accuracy of ± 5 %.

The experiments were not performed under "basal" conditions.

Procedure.

The subject was instructed to avoid exercise and eating at least one hour before the experiments. Upon arrival in the field house with the race track he was seated in a comfortable chair where he rested for 30 minutes, after which he was connected with the spirometer and the respiratory gaseous exchange was recorded for the following

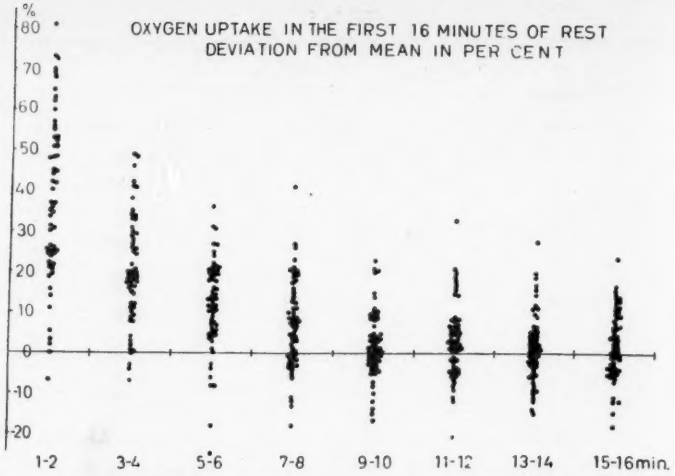

Fig. 1. Oxygen uptake the first minutes after connection with the spirometer, deviation from mean resting values. 59 experiments on 13 subjects.

30 minutes, with the subject still sitting in the same chair. He was then disconnected from the spirometer, walked to the start line, and ran the 70 yards. The subject was connected with the spirometer as fast as possible after the run, and the respiratory gaseous exchange was recorded for the following 40 to 60 minutes.

Five experiments were performed on each of the 13 subjects except subject G, but 4 of the tracings had to be discarded due to technical errors, leaving 59 experiments for consideration.

Results.

The resting values.

The oxygen uptake, carbon dioxide output and pulmonary ventilation were usually higher the first 6 to 10 minutes of the pre-exercise period and were therefore usually determined as the average of the last 20 minutes. Some of the subjects were rather restless and more time was required before reasonable resting values were obtained. See Fig. 1.

After the exercise, the oxygen uptake, carbon dioxide output and pulmonary ventilation were increased for some time, but after 20 minutes the pre-exercise resting values were obtained in all the experiments.

Table II.

Resting O_2 uptake in ml/min. ATPS; and mean resting metabolism before sprint in per cent of basal metabolism.

Subject:	A	B	C	D	E	F	G	H	I	J	K	L	M
1st run:													
Before sprint	360	325	400	320	280	355	370	295	330	410	355	320	295
After sprint	350	315	365	320	335	320	375	285	295	350	350	345	290
2nd run:													
Before sprint	370	360	335	320	300	365	355	290	290	315	300	295	335
After sprint	350	345	390	290	310	345	330	280	265	300	315	305	305
3rd run:													
Before sprint	365	280	345	320	345	390		290	280	360	320	315	340
After sprint	335	315	390	310	365	380		280	285	335	305	300	335
4th run:													
Before sprint	355	305	365	240	325	340		290	270	310	355	325	340
After sprint	345	320	320	245	340	365		265	285	365	360	315	335
5th run:													
Before sprint	350	320		325	290	345			290		350	295	300
After sprint	340	305		325	310	325			300		355	305	295
Mean:													
Before sprint	360	320	360	305	310	360	360	290	290	350	335	310	320
After sprint	345	320	365	300	330	345	355	280	285	340	335	315	310
$\frac{\text{Resting metabolism}}{\text{Basal metabolism}} \times 100$	128	104	119	114	108	121	112	106	103	116	116	112	110

About 45 minutes after the exercise the subjects usually got restless, and as a consequence the "resting values" increased. The post-exercise resting values were therefore determined as the average of the values obtained between the 20th and the 40th minute after the exercise.

The agreement between the resting values for oxygen uptake and carbon dioxide output before and after the exercise was usually good, and the variations in these values rather small (Tables II and III). The resting oxygen uptake corresponded to metabolic rates from 3 to 28 per cent higher than the predicted basal metabolism (table II) calculated according to the standards of Du Bois (1916) and Lusk (1928).

In the 59 experiments in Table II the difference in resting oxygen uptake per minute before and after the exercise was

Table III.

Resting CO_2-output ml/min. ATPS.

Subject:	A	B	C	D	E	F	G	H	I	J	K	L	M
1st run:													
Before sprint	315	260	345	280	275	335	350	250	300	305	320	275	275
After sprint	285	255	305	255	260	275	350	235	260	260	315	265	245
2nd run:													
Before sprint	345	310	295	275	265	345	325	275	245	255	250	260	270
After sprint	315	285	290	240	235	280	285	250	230	240	270	240	270
3rd run:													
Before sprint	340	230	285	255	285	360		270	260	280	280	290	290
After sprint	305	265	300	255	280	300		250	245	290	275	290	275
4th run:													
Before sprint	320	275	300	225	315	330		275	265	255	345	295	325
After sprint	310	290	275	215	270	325		245	235	250	330	280	305
5th run:													
Before sprint	325	285		265	255	330			270		330	290	265
After sprint	310	260		255	250	285			260		310	290	260
Mean:													
Before sprint	330	270	305	260	280	340	340	270	270	275	305	280	285
After sprint	305	270	295	245	260	295	320	245	245	260	300	275	270

10 ml or less in 26 experiments, between 10 and 20 ml in 14 experiments, between 20 and 30 ml in 9 and larger than 30 ml in 10 experiments. The resting oxygen uptake was larger before the exercise in 35 experiments, larger after the exercise in 22 experiments and equal in 2 experiments.

In the 59 experiments in Table III, the difference in resting carbon dioxide output per minute before and after the exercise was 10 ml or less in 20 experiments, between 10 and 20 ml in 16 experiments, between 20 and 30 ml in 11 experiments and larger than 30 ml in 12 experiments.

The resting carbon dioxide output was larger before the exercise in 49 experiments, larger after the exercise in 5 experiments and equal in 5 experiments.

This tendency to a decreased carbon dioxide output with an unchanged oxygen uptake in the post-exercise resting period causes a slight reduction in the respiratory quotient. The mean value for the pre-exercise respiratory quotient was 0.89, for the post-exercise resting period it was 0.84.

Table IV.

Resting ventilation (inspiratory) l/min. ATPS.

Subject:	A	B	C	D	E	F	G	H	I	J	K	L	M
1st run:													
Before sprint	8.5	7.8	8.2	9.1	7.3	7.6	10.5	6.8	6.2	8.2	8.8	5.1	6.4
After sprint	7.3	8.4	9.7	8.7	8.0	7.6	10.4	7.1	5.9	7.5	9.5	5.4	6.0
2nd run:													
Before sprint	8.7	10.6	7.4	8.3	7.1	7.1	9.3	6.9	5.2	6.2	8.1	5.5	6.0
After sprint	8.7	10.9	8.0	8.9	6.9	6.5	8.9	6.5	5.1	6.7	9.1	5.6	6.0
3rd run:													
Before sprint	9.2	9.1	8.0	7.6	7.9	8.8		8.1	6.4	7.9	8.2	6.0	7.2
After sprint	8.9	9.6	8.9	8.3	7.8	7.6		8.3	6.4	8.4	8.8	6.1	6.6
4th run:													
Before sprint	7.4	9.1	7.0	6.6	7.7	7.5		6.7	6.7	7.2	9.6	5.8	7.7
After sprint	7.7	9.7	7.5	6.7	7.3	8.1		6.9	6.0	7.4	9.8	6.2	7.3
5th run:													
Before sprint	9.5	10.4		7.7	7.3	7.6			6.5		9.5	5.6	6.2
After sprint	9.1	9.3		8.9	7.1	7.2			6.3		8.9	7.2	6.5
Mean:													
Before sprint	8.7	9.4	7.7	7.9	7.5	7.7	9.9	7.1	6.2	7.4	8.8	5.6	6.7
After sprint	8.3	9.6	8.5	8.3	7.4	7.4	9.7	7.2	5.9	7.5	9.2	6.1	6.5

The pulmonary ventilation was larger before the exercise in 24 experiments, larger after the exercise in 31 experiments and equal in 4 experiments, see Table IV.

The effect of the exercise.

In 48 experiments the time used for the 70 yard sprint was from 9 to 11 seconds. In 5 experiments the time used for the sprint was less than 9 seconds and in 6 experiments it was more than 11 seconds.

The increase in the respiratory gaseous exchange due to the exercise was determined by subtracting the mean of the pre- and post-exercise resting values from the total exchange the first 20 minutes after the exercise.

The relation between the increase in pulmonary ventilation and oxygen uptake is shown in fig. 2, and between increase in ventilation and carbon dioxide output in fig. 3.

The time required for the respiratory gaseous exchange to reach resting values was then determined.

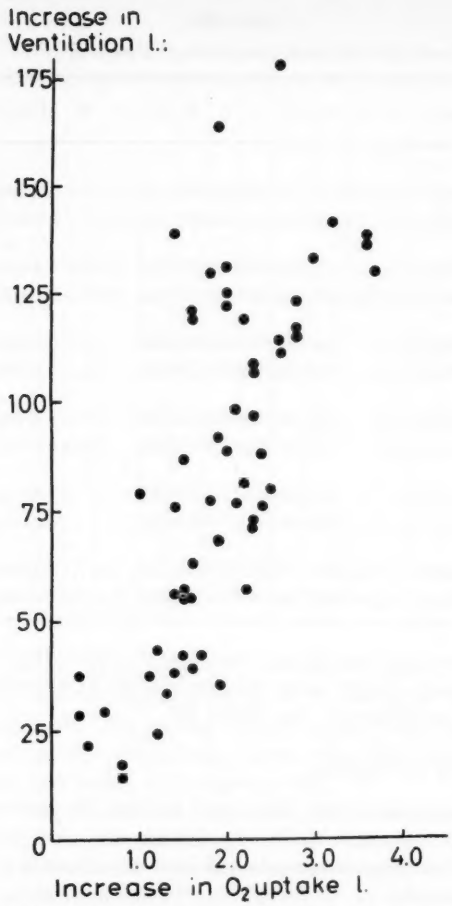

Fig. 2. The relation between increase in oxygen uptake and ventilation in 59 experiments on 13 subjects.

The recovery time for oxygen uptake was found to be shorter than the recovery time for carbon dioxide output, which also was found to vary less in experiments on the same subject.

In table V the data for increase in oxygen uptake, carbon dioxide output and the recovery times are given for the 48 experiments where the time used for the sprint was from 9 to 11 seconds.

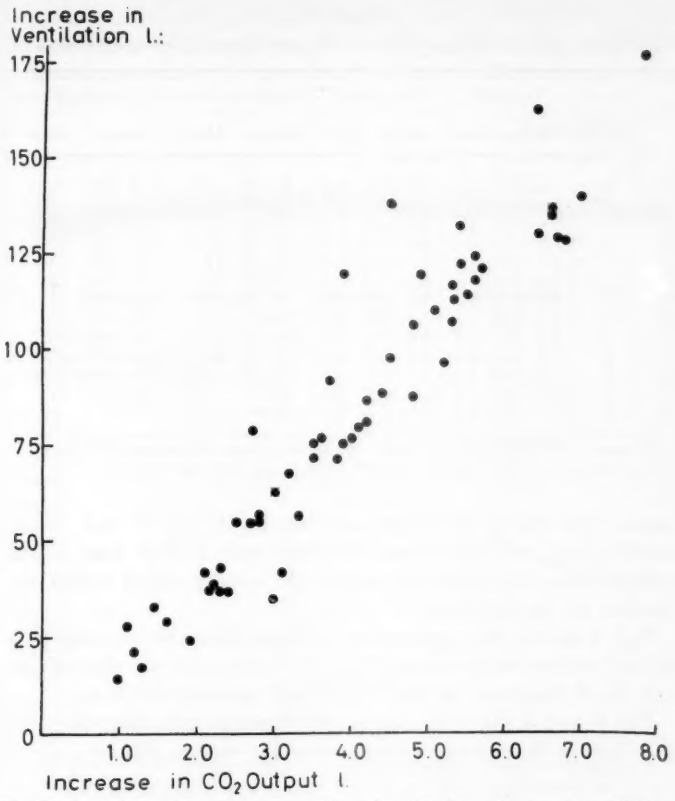

Fig. 3. The relation between increase in carbon dioxide output and ventilation in 59 experiments on 13 subjects.

In the experiments where the subjects were running exceptionally fast or slow, the influence of the work intensity on the respiratory gaseous exchange was marked.

For comparing the recovery times of the different subjects, these experiments have therefore been omitted from table V.

Based on the recovery time for carbon dioxide output, the subjects can be divided in groups. The subjects M and A were well trained, and the results in the following season confirmed this. Of the subjects K, L, J, B, F, G, H and I, only L was considered in good form by the coach, but his results in the following

Table V.

Respiratory gaseous exchange after a 70 yard sprint in 9.0 to 11.0 seconds.

Subject	No. of experiments	"O_2-debt"		"CO_2-excess"		O_2-recovery time		CO_2-recovery time	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
A ..	4	1.4—2.0	1.7	2.1—4.4	2.9	6—12	8 1/2	8—12	9 1/2
B ..	4	1.4—2.3	1.8	3.2—3.9	3.6	8—14	11 1/2	10—16	12 1/2
C ..	3	1.4—1.9	1.6	4.5—6.4	5.3	8—12	10	14—16	15
D ..	5	2.0—3.6	2.7	4.8—6.6	5.6	6—16	11	14—16	15
E ..	3	2.1—3.6	2.9	4.5—6.6	5.5	6—14	12	12—18	15
F ..	5	1.8—3.2	2.3	5.2—7.0	6.0	8—18	12	10—14	13
G ..	2	1.4—1.9	1.7	2.8—3.0	2.9	6—10	8	12—14	13
H ..	2	1.5—2.2	1.9	3.3—5.6	4.3	8—8	8	12—14	13
I ...	5	1.6—2.5	2.2	3.0—4.8	3.9	10—14	12 1/2	12—14	13
J ..	4	1.9—2.6	2.2	2.8—6.4	4.6	8—14	11	8—16	12
K ..	5	1.0—2.6	1.7	2.5—5.1	3.6	4—12	6 1/2	10—14	11
L ..	3	0.8—1.5	1.2	1.0—3.1	2.0	6—8	6 1/2	10—12	11
M ..	3	0.3—0.8	0.6	1.3—2.3	1.7	4—6	5 1/2	8—8	8

Oxygen debt and carbon dioxide excess are given in liters at ATPS.
Oxygen and carbon dioxide recovery times are given in minutes.

season were not good. Three subjects, C, D, and E, had exceptionally long recovery times. Of these, only D took part in the competitions the following season. He usually ended rather exhausted as the last man.

Fig. 4 shows the spirometer tracings after the exercise of a subject with a short recovery time for carbon dioxide elimination, and fig. 5 diagrams of the respiratory gaseous exchange.

Fig. 6 and 7 show the spirometer tracings and diagrams from a subject with a long recovery time for carbon dioxide.

Discussion.

The experiments described had to be carried out when the students were available, and the conditions were therefore not basal. It was found that after being connected with the spirometer for 6 to 10 minutes the respiratory gaseous exchange reached a rather steady resting level. The mean resting oxygen uptake of the different subjects corresponded to a metabolism which was from 3 to 28 per cent higher than the predicted "basal metabolism".

Benedict and Cathcart (1913) found that subjects under basal conditions had an increment of their metabolism of 6.1 to 24.8 per cent by changing from the lying position to sitting

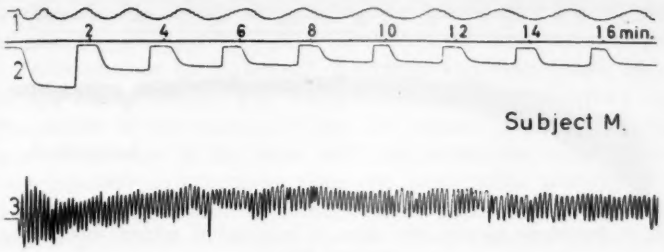

Fig. 4. Spirometer tracings after sprint of a well trained subject with short recovery time.

1. Ventilation.
2. Carbon dioxide output.
3. Respiratory difference curve.

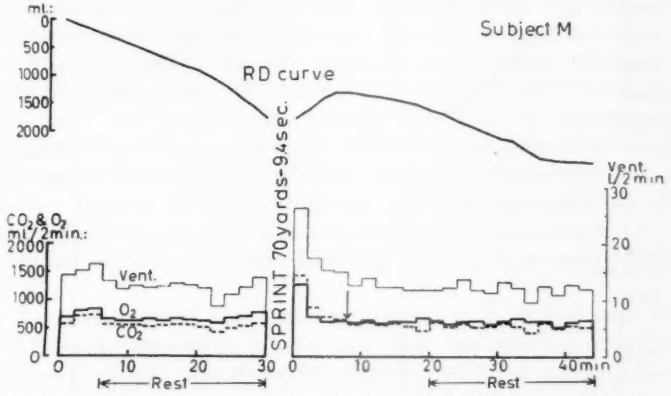

Fig. 5. The respiratory gaseous exchange before and after sprint of a well trained subject.

inactive on a bicycle ergometer. It would therefore seem that the advantage of having basal conditions under these experiments would have been rather uncertain.

HILL, LONG and LUPTON (1924) found that the respiratory gaseous exchange returned to resting values few minutes after completion of exercise (running) when this did not result in a marked increase of the lactic acid concentration in the blood. The fast return to resting level of the oxygen uptake after moderate exercise was verified by SIMONSON (1926), who also found that training increased the speed of recovery.

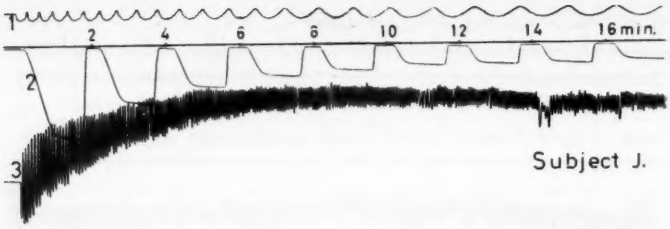

Fig. 6. Spirometer tracings after sprint of an untrained subject with a long recovery time.

1. Ventilation.
2. Carbon dioxide output.
3. Respiratory difference curve.

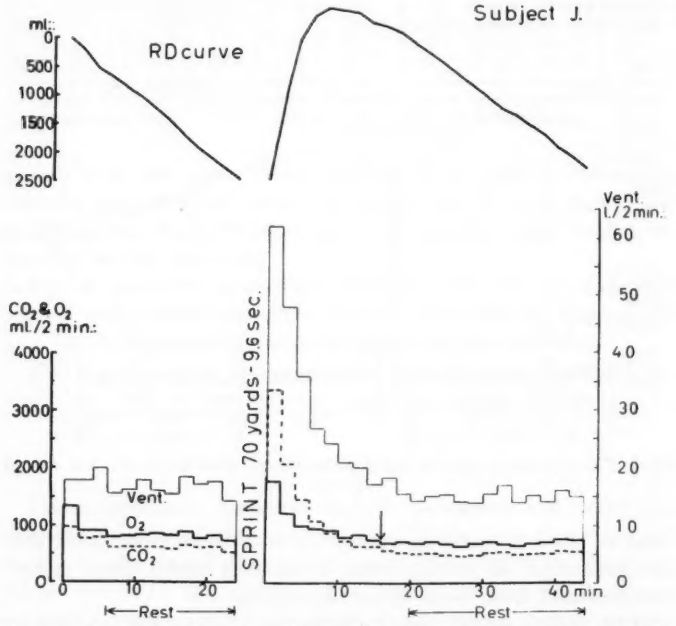

Fig. 7. The respiratory gaseous exchange before and after sprint of an untrained subject.

LIEBENOW (1928) and HEBESTREIT (1929), using running in staircases and knee-bending exercises, found that after moderate exercise the resulting increase in oxygen uptake and carbon dioxide output were both exponential functions of time, the

carbon dioxide output attaining resting values after the oxygen uptake.

Investigations by MARGARIA, EDWARDS and DILL (1933) confirmed these findings and found almost complete recovery in the course of few minutes when the exercise did not lead to marked increase of the lactic acid (alactic oxygen debt) and a slow recovery (up to more than one hour) when the exercise was sufficiently heavy to incur a lactic acid oxygen debt (in their experiments an oxygen uptake higher than 2.4 l/min.).

All these workers determined the respiratory gaseous exchange by fractionate collection of the expired air in bags, in which the oxygen percentage and by some also the carbon dioxide percentage were determined by volumetric methods.

BERG (1948) confirmed the results of the previous workers, utilizing a concentration analyzer for determining the oxygen- and carbon dioxide percentage in a fraction of the expired air. A 3 minute step-up exercise resulting in an oxygen uptake of 800 to 1,100 ml/min. led to a recovery time of 6 to 10 minutes. He found that the half-time recovery constants for oxygen and carbon dioxide showed definite interindividual differences, that the oxygen debt is paid considerably faster than the corresponding carbon dioxide excess is eliminated, and that the reliability of the carbon dioxide time constant was high, of the oxygen time constant considerably lower. Training reduced the recovery constants.

Investigations of the respiratory function in diving animals by SCHOLANDER (1940) demonstrate that the recovery time for oxygen is shorter than for carbon dioxide also in this comparative material.

The experiments described in this paper confirm the findings in these previous investigations, even when the exercise is of as short duration as approximately 10 seconds. The carbon dioxide output regained resting values slower than the oxygen uptake. The recovery time for the carbon dioxide was a more constant characteristic for each individual than the recovery time for the oxygen.

Running 70 yards in approximately 10 seconds resulted in an oxygen debt, which compared with the values reported by MARGARIA et al., is of a magnitude where a marked increase in lactic acid in the blood and a slow recovery rate, can be expected. It seems reasonable to assume that the prolonged recovery times

of some of the subjects are due to a lactic acid oxygen debt, to which the reduced resting carbon dioxide output after the work also points. The small difference in pre- and post-exercise carbon dioxide output, only about 5 per cent, indicates that the increase in lactic acid in the blood has been rather slight.

In spite of the varying work intensities and lack of observations during, and the first seconds after the work, good correlation was found between the recovery times for carbon dioxide and the later achievements of the test subjects in track competitions.

Summary.

Spirometric investigations of the respiratory gaseous exchange during resting, not basal, conditions and the recovery after a 70 yard sprint are reported.

It is shown that around 10 minutes after connection with the spirometer fairly constant values for oxygen uptake and carbon dioxide output were obtained. These resting values were regained shortly after the exercise. The time used for elimination of excess carbon dioxide was longer and more constant, and showed greater interindividual differences than the time required for covering the oxygen debt.

Well trained subjects with good results in track competitions showed the shortest recovery times. Trained subjects with fair to poor results had recovery times overlapping the recovery times of untrained subjects in good physical condition.

Acknowledgements.

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Recorder for Blood-Flow Determination.

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When recording the arterial blood flow, it is often desirable to have an apparatus which permits direct determination of the exact flow per time unit. Moreover, it is expedient on practical grounds for the blood flow to be recorded directly on the kymograph curve. The apparatus described in the following meets these requirements, and allows direct determination of the blood flow per 0.5 to 2 seconds. In its present state, it has been used chiefly for recording the blood flow in the abdominal aorta of the anesthetized cat.

Apparatus.

Principle. The principle of the apparatus can be inferred from the sketch in Fig. 1. The blood flow from I to II is occluded by means of an electromagnetically actuated tube clamp, III, for a fixed interval (0.5 — 2 sec.). This causes the blood to act on a rubber membrane, IV. Owing to displacement of the membrane and the volume of blood on the other side of it, the blood flow to peripheral areas is uninterrupted. The displacement of the membrane is recorded on smoked paper by the needle, V.

Apparatus. On practical grounds, the apparatus is in two sections. The arrangement for occluding the blood flow is shown in Fig. 2, and the recording apparatus with the movable rubber membrane in Fig. 3.

The blood flowing from the central part of an artery is led, through plastic tubing, to the glass tube, *E* (Fig. 2). When the magnetic valve, *G*, is open, the blood runs via the plastic tubing *H* and tube *F* to the

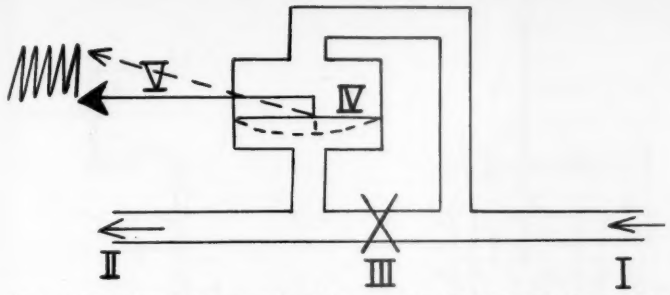


Fig. 1. *Sketch showing the principle of the apparatus for blood-flow determination.* The apparatus is connected to an artery so that the blood flows from I to II. III is an electromagnetically actuated clamp. IV is a rubber membrane, and V a lever for recording the movements of the membrane.

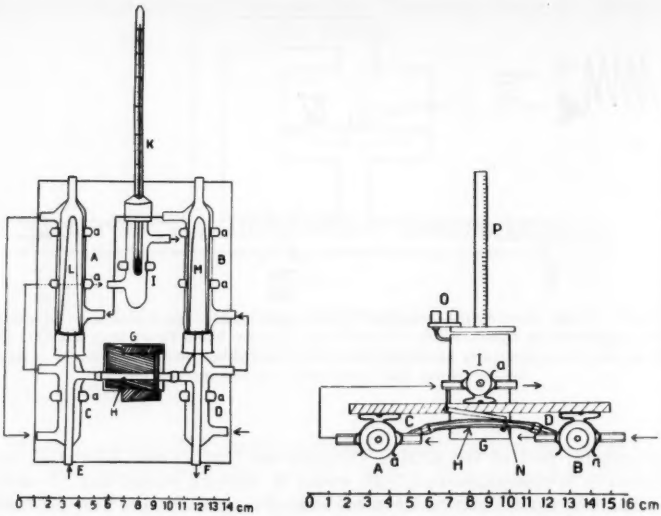
peripheral part of the artery. Through one limb of the T-tube *E*, the blood is in communication with vessel *A*, where a rubber bag, *L*, separates it from the fluid on the other side of the membrane. This prevents the blood from being diluted by the fluid present in the apparatus. Tube *F* communicates in the same way with vessel *B*. The vessels and tubes *A*, *B*, *C* and *D* are surrounded by glass jackets, through which warm water (37°) runs, starting to the right in vessel *D*, and finally passing the control thermometer, *K*, in vessel *I*. This prevents the blood from being chilled during its extracorporeal passage. The fluid in vessels *A* and *B* is in communication with tubes *F* and *E*, respectively, through plastic tubing filled with fluid (Fig. 3).

Fig. 2 b is a horizontal section of the apparatus. *P* is a metal rod for attaching the apparatus, which is mounted on a plastic slab. *O* marks the contacts to the electromagnet.

The electromagnet, *G*, which closes and opens the passage in the rubber tube *H*, is regulated by electric impulses from a timer. This is set so that tube *H* is clamped at fixed intervals (5 or 10 sec.) for a period ranging from 0.5 to 2 sec. This period is regulated to obtain suitable discharges.

The recording apparatus shown in Fig. 3 is of plastic, and consists of three parts, *A*, *B* and *C*, joined together by the rods *D* with the nuts *G*. The different parts are kept tight by rubber packings.

Tube *E* in the lower part of *A* opens into a funnel-shaped widening, which is covered by the rubber membrane *O*. A strip of plastic, *R*, is glued to the centre of the rubber membrane. Through this passes the rod, *K*, leading to one end of the plastic rod, *I*. The rod is kept in position by a thin steel needle through *K* and *R*. The forked rod *K* is joined by a wire loop to rod *L*, of which the continuation is provided with a pen for recording the movements on the kymograph. Leakage at the exit of rod *L* at *H* is prevented by a polyethylene membrane, *P*, attached by screws between *N* and *O*. Owing to the properties of this membrane, a hole with



Figs. 2 a and b. *Arrangement for recording the blood flow.* Fig. 2 shows the part of the apparatus which occludes the direct blood flow for its recording via the movable rubber membrane in the apparatus shown in Fig. 3.

a "collar" is formed in the plastic when a rod with a finely ground tip, such as rod *L*, is introduced through it. This ensures absolute freedom from leakage round rod *L*, without hampering its movements. This leakproof passage is the most important technical refinement of the apparatus.

Before use, both apparatuses and their tubes are filled with physiologic saline, and all air bubbles evacuated. Heparin is added to the saline in tubes *E*, *H* and *F* and the appurtenant internal parts of the rubber bags *L* and *M* (Fig. 2). The apparatus shown in Fig. 2 is placed as close as possible to the artery from which the recording is to be made, to avoid chilling of the blood in the relevant tubes.

Function. The apparatus functions as follows. As long as the magnetic valve *G* is open, the blood passes directly from *E* to *F* via *H* (Fig. 2 a). The tension in the rubber membrane *O* (Fig. 3) keeps it in a "neutral" position. When tube *H* is clamped by the magnet *G*, the blood flow through it is stopped, and the blood is forced into the rubber bag *L*, which in turn causes displacement of the fluid on the other side of the rubber membrane. The pressure is transmitted from vessel *A* (Fig. 2) via tube *F* (Fig. 3) to membrane *O*, which is depressed, and displaces the same volume of fluid from *A* (Fig. 3) to vessel *B* (Fig. 2). This

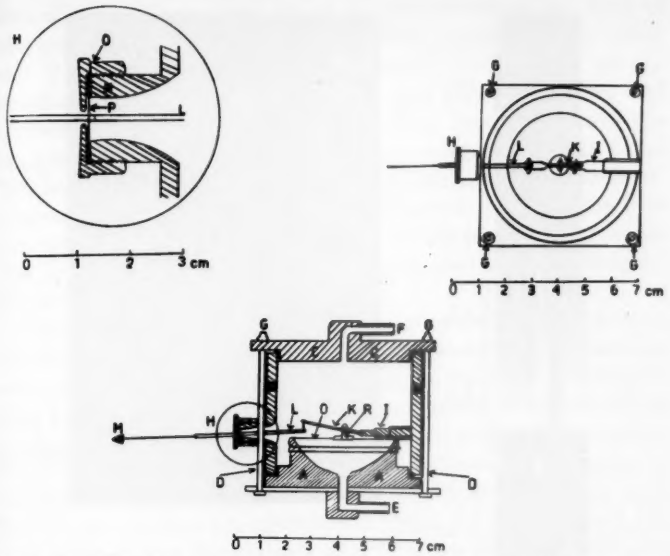


Fig. 3. *Apparatus for recording the blood flow.* Lower centre: vertical projection. To the right above: horizontal projection. To the left above: enlargement of detail *H*. For description, see text.

implies that exactly the same volume of blood forced into the rubber bag *L* (Fig. 2) is expressed from the rubber bag *M* (Fig. 2) into the peripheral part of the blood vessel. By this means, the blood flow is uninterrupted. The bulging of the rubber membrane *O* (Fig. 3) is dependent on the quantity of blood flowing from the central part of the artery during the time tube *H* is clamped. The greater the blood flow, the more does the membrane bulge. When the membrane is depressed, it actuates the lever system *I*—*K*—*L*, and the pen *M* is raised in proportion to the blood flow. When the magnet thereafter releases the flow via tube *H* (Fig. 2), the rubber membrane *O* (Fig. 3) and the pen *M* return to the neutral position. There is concurrent displacement of fluid and blood from vessel *B* to vessel *A* (Fig. 2). The initial position has then been regained, and on the next clamping of the tube the procedure just described is repeated.

The apparatus is easily standardized. To do this, tube *H* is clamped, and rising quantities of fluid are introduced through

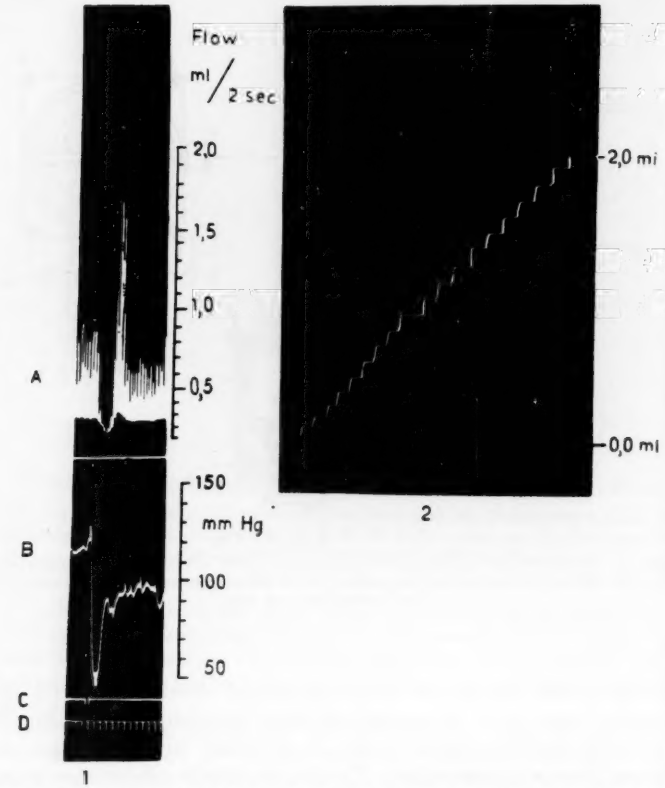


Fig. 4. *Recording of the blood flow in the abdominal aorta of the eviscerated cat.* Weight of animal 2.9 kg. Dial anesthesia. *A* denotes the blood flow per 2 sec. Determinations at 9-sec. intervals. B = blood pressure. C = injection signal. D = time marking, 10 sec. At 1, injection of 10 μg acetylcholine. On upper right (2) a standardization curve. Each "step" denotes a volume of 0.1 ml.

E (Fig. 2). It is advisable to start with 0.1 ml and to increase the total volume by 0.1-ml steps until 2.0 ml is reached.

Fig. 4 illustrates both the results of determination of the blood flow in the abdominal aorta of the eviscerated cat, and standardization of the apparatus.

Discussion.

Several apparatuses have earlier been described which allow direct and exact determination of the blood-flow volume in rela-

tion to time. Among them are those constructed on the basis of the original designs of VOLKMANN (1850), LUDWIG (1867, 1895) and PAWLOW (1887). Examples of such modifications of the stromuhr have been reported by LU and MELVILLE (1950) and DAWES, MOTT and VANE (1952). All these flowmeters have the common property of measuring the time required for the blood flow to reach a certain, fixed volume. In many cases it is, however, of greater interest to obtain direct information regarding the flow per time unit. Moreover, it is then easier to determine rapid changes in blood flow. The apparatus described here was designed on the basis of these considerations.

The apparatus is exceedingly reliable in operation, and has been run for many hours at a stretch in the experiments. We have never observed fibrin or blood clots in either the tubes or rubber bags after heparinization of the animal. The resistence to the blood flow produced by the rubber membrane seems to be of no importance. The pressure required to displace this membrane is negligible in comparison to the arterial blood pressure. As can be inferred from Fig. 4, the baseline becomes shifted, owing to the fact that the time between two determinations will be too short to allow the membrane to return to the neutral position if the flow is large. Calculation of the blood flow is performed by measuring the size of the discharges. Consequently, it is uninfluenced by the aforementioned shift in the baseline.

Summary.

An apparatus is described for direct, exact determination of the blood flow in large arteries of anesthetized animals. It permits direct recording on the kymograph curve of the blood flow per time unit (0.5 — 2 sec.).

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Cortical Reception of Cold Impulses from the Tongue of the Cat.

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In a previous investigation COHEN, LANDGREN, STRÖM and ZOTTERMAN (1957) found, in the cortical tongue projection area, cells responding to cooling of the tongue. Only two cells were observed one of which responded to 0.1 M acetic acid as well as to cooling. It was considered of interest to collect some more information concerning the cortical "cold" cells and to study their specificity to the stimulus and the pattern of their impulse discharge. This was done in a series of experiments, which will be described here.

Methods.

Ten cats were used as experimental animals. The anaesthesia during the operation was Intraval (thiopentone sodium) administered intraperitoneally at a dosage of 40 mg/kg body weight. When necessary additional injections of the same anaesthetic were made intravenously. When the operation was finished a local anaesthetic (xylocain 2 % with exadrin "Astra") was applied extradurally to the exposed second cervical segment. Local anaesthesia was applied to the pressure points of the head holder as well as to the incisions in the cranial area. The experiments were done 4—15 hours after the last Intraval injection. The xylocain block applied to the spinal cord was obviously only partial, as spontaneous respiration was not abolished and it was generally not necessary to give artificial respiration. The partial block was, however, effective enough to keep the animal motionless with relaxed extremities. The vasomotor tone and the circulatory conditions in general were less interfered with in these animals than in animals with high spinal section.

The surgical approach to the cortical tongue projection area was the one described by COHEN, LANDGREN, STRÖM and ZOTTERMAN (1957) as the indirect approach *i. e.* the microelectrode was inserted at the lateral end of s. cruciatus and penetrated blindly in the ventro-lateral direction about 10 mm down to the tongue area. An approximate localization of the microelectrode track was achieved by a final exposure of the frontal part of the hemisphere and penetration with the microelectrode left in place after the last experimental track. The tip could then be observed to penetrate within the tongue projection area. The cortical potential evoked by electrical stimulation of the lingual nerve or of the tongue surface directly was used as a physiological means of localization. Having localized the responsive cortical area the trephine hole in the skull was closed by a perspex disc cemented to the edges of the bone with dental cement. The microelectrode was inserted through a small hole in the disc about 1 mm in diameter. The glossopharyngeal and hypoglossal nerves were sectioned bilaterally in order to prevent any reflex contraction of the tongue induced by the applied stimuli.

Recording and electrical stimulation was done according to the technique described by COHEN, LANDGREN, STRÖM and ZOTTERMAN (1957). The cortical cells were tested with the following physiological stimuli:

Mechanical Stimuli.

Light touch of the tongue surface. Pressure on the tongue by a glass rod. Stretching of the tongue. Movements of the jaw. The effect of spontaneous tongue movements and swallowing was observed.

Thermal Stimuli.

Water and Ringer's solution of various temperatures between 5 and 45°, which were applied to the tongue through a funnel with a stopcock. The latter was connected to a microswitch which applied a small voltage step to the signal beam of the CRO. The time lag of the signal was tested and corrections were made when the records were evaluated.

Taste Solutions.

0.5 M NaCl
0.01 M Quinine HCl in Ringer's solution
0.3 M Acetic acid in Ringer's solution (PH = 3)
Distilled water.

These solutions were applied in the same way as the thermal stimulis All taste solutions were applied at a temperature of 30° which wae approximately the temperature of the tongue surface under these experimental conditions.

Impulse frequency of the cortical cell discharge was counted for 0.5 second intervals. Negativity in all figures is recorded upwards. All action potential records shown are extracellular, as no resting potential was observed.

Results.

Of 325 cortical cells, tested with mechanical, thermal, gustatory and electrical stimuli applied to the tongue of the cat, 36 cells responded to cooling of the tongue. Among these 36 cells a group of 12 cells responded to cooling only and not to the other physiological stimuli used. Some of them were, however, discharged by electrical stimulation of the tongue. Another group consisting of 17 cortical cells responded to cooling and to mechanical stimulation. Three cells responded to cooling, warming and mechanical stimulation, three to cooling, warming, taste and mechanical stimuli and finally one cell responded to cooling, taste and mechanical stimuli.

The response of a cortical cell to cooling of the tongue is shown in Fig. 1. Record A is a control, water of 37° being flushed onto the tip of the tongue at the signal on the upper tracing. In B and C cold water (11°) was applied. The cortical cell responds to cooling of the tongue with a series of action potentials lasting 0.5 — 1 sec. The frequency of the discharge is irregular and the spikes often appear in groups with high frequency. The cell was spontaneously active at a frequency of less than 1 spike per sec. During the first second of the response about 30 spikes were discharged.

The cell shown in Fig. 1 is of particular interest for two reasons: firstly because of the short latency of the response to cooling and secondly because it belonged to a group of cortical cells discharged only by cooling of the tongue and was inaccessible to other physiological stimuli. The latency of the response to cooling of the tongue was about 20 msec. As the cell was discharged by electrical stimulation within the receptive field on the tip of the tongue, it was possible to study the response on a faster time scale. Fig. 10 shows that the cell was fired with a shortest latency of 14 msec. The first spikes appear on the falling phase of the primary cortical potential, which in this cortical layer is negative in sign. As shown by Cohen, Landgren, Ström and Zotterman (1957) the primary evoked potential in the cortical tongue projection area is produced by the afferent volley in fast conducting fibres from the touch receptors of the tongue. Cortical cells of this area responding to touch of the tongue are discharged with a shortest latency of 5 msec., when the tongue is stimulated electrically. They appear on the rising phase of the primary potential. The shortest latency

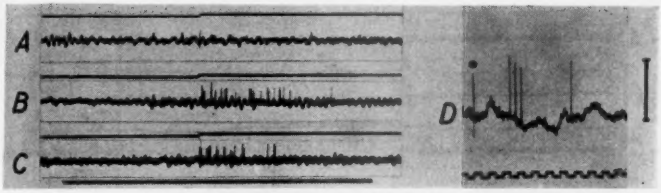

Fig. 1 Response of cortical cell to cooling of the tongue. A. water 37°, B and C water 11° applied to the tongue. Signal on top beam. Time: 50 c. p. s. D. the same cell responding to electrical stimulation of the ipsilateral tip of the tongue. Time: 5 msec. Voltage scale 1 mV.

of the "cold" cells is thus about three times that of the "touch" cells. Approximately the same relationship is true for the conduction velocities of the touch and cold fibres from the tongue, the former is about 50 m/sec. and the latter about 20 m/sec. (*c. f.* ZOTTERMAN 1936 and COHEN, LANDGREN, STRÖM and ZOTTERMAN 1957).

As far as can be judged from these experiments the cortical cell of Fig. 1 was a "specific cold cell". It did not respond to warming, nor to any of the taste solutions and not to touch, pressure or stretch applied to the tongue.

The response to cooling of the tongue varied greatly between different cortical cells but each cell gave a reasonably constant type of discharge. Fig. 2 gives the four essential types of cold responses observed in the group of cortical cells studied. Record A is from the "specific cold cell" described above. The response is phasic, of short latency and relatively high frequency. The responses of two other cortical cells discharging only to cooling of the tongue are shown in Fig. 2 B and C. The response shown in B has a long latency (300 to 600 msec.) and consists of a short series of spikes at a low irregular frequency. As in A the cell is spontaneously active but at a very low frequency (below 1 imp./sec.). It was also possible to fire cell B by electrical stimulation of the tongue. To this type of stimulus it responded with a short latency of 8 msec. which indicates that the cell is accessible to impulses conducted in fast afferent fibres. This latency is of the same order of magnitude as that of cortical "touch cells" discharged by electrical stimulation of the tongue (5 — 8 msec.) but the cell did not respond to touch, pressure or stretch applied to the tongue.

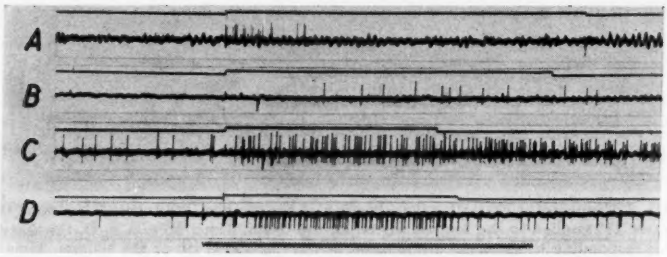

Fig. 2. Different types of impulse discharge recorded from cortical cells responding to cooling of the tongue only (A—C) and to cooling and touch (D). Time: 50 c. p. s. The signal on the top beam marks the application of Ringer's solution at 10° to the tongue.

Cell C of Fig. 2 showed a spontaneous activity of relatively high frequency. The response to cooling of the tongue using Ringer's solution at 10° was very vigorous. The time course of this response is further illustrated in Fig. 3. The maximal frequency of the response was not reached immediately. During the first second after the beginning of the cooling the frequency of the discharge rose from about 10 imp./sec. to 40 imp./sec. Between one and two seconds after the beginning of the stimulation a maximal frequency of 60 imp./sec was observed. The cooling of the tongue only lasted for about one second and the maximal frequency of the cortical discharge thus appeared during the phase of declining stimulus intensity.

Fig. 3 shows another interesting feature often observed in cortical "cold cells". As can be seen from the control interval before cooling the spontaneous activity shows a tendency to rhythmic variations in frequency. The vigorous response to cooling exaggerates the rhythmic variations and the cold response is followed by a series of grouped discharges lasting more than 15 seconds. Each of these repeated bursts of impulses lasts for about one second and consists of 9 — 16 spikes per group. Between the groups there is an interval of about 2 seconds in which no spikes are fired. The characteristic features of the type of cortical response to phasic cooling of the tongue shown by cell C of Fig. 2 thus are first, a high frequency initial discharge with a gradual increase in frequency over the first two seconds of the response and lasting longer than the cooling; second, a repetitive after-discharge with bursts of spikes reappearing at a regular rhythm.

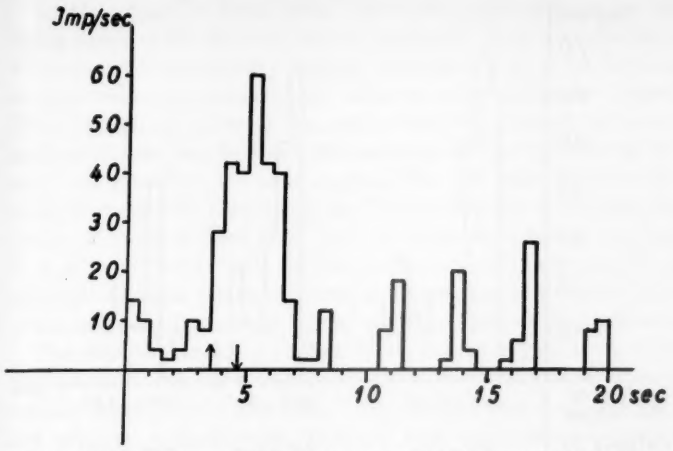

Fig. 3. Impulse frequency diagram of the discharge of the cortical cell, shown in Fig. 2 C, in response to cooling of the tongue. Ringer's solution at 10° applied between arrows. Note the gradual build up of the impulse frequency and the rhythmic bursts of after-discharge.

Finally Fig. 2 D shows a cortical cell which responds to cooling of the tongue with a long lasting increase in the spontaneous frequency of discharge which in this case was about 1 imp./sec. The actual cooling elicited a high frequency discharge with a latency of 80 msec. The maximal frequency (70 imp./sec.) was reached during the first second of the response and there was no gradual increase in frequency as in the cell of Fig. 2 C. When the cooling ceased the frequency of the discharge during the rewarming of the tongue fell considerably but remained between 10 and 20 imp./sec. during more than 5 seconds. This type of response thus differs from the above described phasic and rhythmic ones. The cell seems to behave like the afferent cold fibres of the lingual nerve (HENSEL and ZOTTERMAN 1951) responding at a higher rate as long as the temperature of the tongue is below a certain level.

Cell D of Fig. 2 also responded to touch of the ipsilateral tip of the tongue within a well defined receptive area. Electrical stimulation of the tongue discharged the cell with a latency of only 4.5 msec. showing that the cell is accessible to afferent impulses in fast conducting fibres.

The latency, frequency and duration of the cortical "cold cell" discharge was dependent on the intensity of the stimulus. This

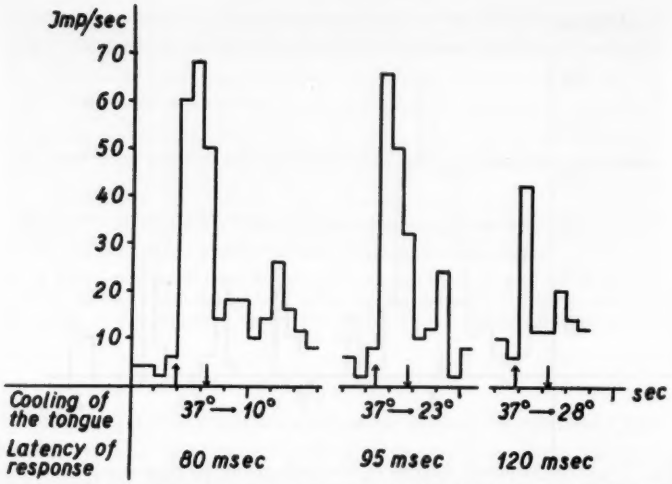

Fig. 4. Impulse frequency diagram showing the relationship between the intensity of the stimulus and the response of a cortical cell to cooling of the tongue.

is shown by Fig. 4 which gives the impulse frequency of a cortical cell responding to cold water of three different temperatures. Before the application of the cooling the tongue was warmed with Ringer's solution of 37° C. Thus cooling from 37° to 10° elicited a response with a latency of 80 msec. and a maximal frequency of 68 imp./sec. (counted on 0.5 sec. intervals). The frequency remained above 50 imp./sec. during 1.5 seconds. Cooling from 37° to 23° gave a latency of 95 msec. and a maximal frequency of 66 imp./sec. the frequency remaining above 50 imp./sec. for 1 second. For a cooling from 37° to 28° the parameters were 120 msec. 42 imp./sec. and 0.5 sec. respectively.

Discussion.

The cortical cells responding to cooling of the tongue may be devided into two groups: those responding only to cooling and those responding also to other types of physiological stimuli applied to the tongue. In the first mentioned group are cells responding with a short latency. A comparison between the latencies of "specific" cortical "touch cells" and those of the fastest responding "specific cold cell" shows that the latter is about 3 times longer than the former. The same relationship holds true for the conduction velocities of the peripheral part of the two afferent paths.

In the "specific touch cells" the first spike appears on the rising phase of the primary evoked potential. They may therefore be assumed to be primary cortical touch cells *i. e.* to be the first cortical cells responding to the afferent volley from the tongue. The relationship between the conduction velocities of the touch and cold fibres and between the latencies of the "specific touch" and the "specific cold" cells suggests that the latter are also primary cortical cells thus being the first to respond to the afferent volley in the cold fibre path. The existence of "specific" cortical "touch" and "cold" cells further indicates that the two types of afferent impulses can be conveyed to the cortex in separate paths without mixing in the relay nuclei of the medulla or the thalamus.

The cells discharged by cooling of the tongue as well as by other physiological stimuli always responded to cooling with a long latency usually about 100 msec. They are therefore most certainly not primary cortical cells. Cells of this type will be further described in a following paper.

Summary.

Cortical cells within the tongue projection area were studied with extracellular microelectrode recording technique.

Thirty-six cortical cells responded to cooling of the tongue; 12 of these cells responded to cooling only whereas 24 cells were discharged also by other physiological stimuli mainly by touch or pressure applied to the tongue.

Four different patterns of "cold cell" discharge is described.

The latency, duration and frequency of the cortical "cold cell" response was observed to be dependent on the intensity of the cooling of the tongue.

Cells responding with short latency (15 — 20 msec.) to cooling were found within the group of cortical cells responding to cooling only. In the group of cells discharged by cooling as well as by other stimuli the response to cooling showed a long latency (about 100 msec.).

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Convergence of Tactile, Thermal, and Gustatory Impulses on Single Cortical Cells.

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Cortical cells in the cat's tongue projection area responding only to tactile, only to thermal or only to gustatory stimuli applied to the tongue were described in previous reports (COHEN, LANDGREN, STRÖM and ZOTTERMAN 1957, LANDGREN 1957). Among the cortical cells activated by cooling of the tongue there was, however, a relatively large group of cells also discharged by tactile stimulation. The following investigation deals with cells of this type *i. e.* with cortical cells responding to impulses in afferent fibres subserving different sensory modalities.

Methods.

The experimental technique was described previously (COHEN, LANDGREN, STRÖM and ZOTTERMAN 1957, LANDGREN 1957). Briefly, the activity of single cortical cells was recorded extracellularly with the conventional microelectrode technique. The cortical tongue projection area on the orbital surface of the hemisphere was reached by penetration with the microelectrodes from the lateral end of the cruciate sulcus. The stimuli were applied directly to the tongue and were mechanical, thermal, gustatory or electrical in nature. Several types of mechanical stimuli were used. They were touch or pressure on the tongue with a small glass rod, stretching the tongue, moving the lower jaw, spontaneous tongue movements and swallowing.

The taste solutions used were 0.5 m NaCl, 0.01 M Quinine HCl in Ringer's solution, 0.3 M acetic acid in Ringer's solution (PH 3) and distilled water. Cooling and warming of the tongue was accomplished by flushing water or Ringer's solution of desired temperature onto the tongue.

In most of the experiments the hypoglossal and the glossopharyngeal nerves were sectioned bilaterally to eliminate reflex movements of the tongue.

Intraval (Thiopentone sodium) anaesthesia was used during the operation. During the experiment, which was carried out 4—15 hours after the last intraval injection, a local anaesthetic block was applied to the exposed second cervical segment of the spinal cord. Wounds and pressure points in the head area were locally anaesthetised.

Results.

Material.

In 10 experiments 609 cortical cells were observed. It was possible to record from 325 of these cells for a time long enough to allow proper testing with the above mentioned stimuli. All tested cells showed spontaneous activity of varying frequency and about $^2/_3$ of them did not respond to any of the stimuli used. However, 101 cortical cells were discharged by one or more than one of the test stimuli. The effective stimuli and the number of cells responding are listed in table I.

Table I.

Stimulus applied to the tongue	Number of responding cortical cells
Touch	32
Stretch[1]	29
Cooling	12
Warming	1
Cooling and touch	8
Cooling and stretch[1]	9
Cooling, warming and touch	3
Cooling, warming, taste and stretch[1]	3
Cooling, taste and stretch[1]	1
Taste and stretch[1]	1
Taste and touch	2
Total	101

[1] Stretch stands for a group of different stimuli assumed to affect stretch receptors in the tongue pharyngeal and (or) masticatory muscles. The stimuli were: stretching the tongue, tongue movements, pressure on the tongue, swallowing and movements of the lower jaw.

The Response to Touch and "Stretch".

The two largest groups of cells responded to mechanical stimuli only. These cells were divided into two groups on the basis of the type of stimulus discharging them. Those responding to light touch with the rod within clearly circumscribed areas of the tongue surface were recorded in one group, while cells requiring hard pressure within less well defined tongue areas were listed together with those responding to stretch of the tongue, movements of the lower jaw, spontaneous tongue movements or swallowing. It was assumed that the last mentioned group of stimuli, for convenience referred to below as "stretch", affected stretch receptors in the extrinsic tongue muscles (cf. COOPER 1953), masticatory or pharyngeal muscles.

The observation of a large number of cortical cells responding to tactile stimuli only, is in agreement with our earlier findings (cf. COHEN, LANDGREN, STRÖM and ZOTTERMAN 1957). There were certain differences in the cellular response to touch and stretch of the tongue. The response to touch consisted of a series of impulses of relatively high frequency and closely related to the slow cortical evoked potentials (Fig. 1 A). "Stretch", however, often gave rise to a gradual and moderate increase of the spontaneous frequency of the cortical cell. The impulse discharge was not always related to the evoked potential. This type of discharge is illustrated in Fig. 1 B which was, however, recorded from a cortical cell responding not only to "stretch" but also to cooling of the tongue. Both types of mechanical stimuli discharged impulses in afferent fibres of high conduction velocity, as judged from the short latency of the cortical response. Touch as well as stretch receptors discharged cortical cells with a latency of 5—8 msec. when electrically stimulated by a shock applied to the tongue surface. An adequate analysis of these two types of cortical discharge requires further investigations. The most important point for the present purpose is that the findings strongly suggest the existence of cortical cells affected specifically by one quality of sensation.

The cortical cells responding to cooling of the tongue were treated in detail in a previous report (LANDGREN 1957). In this case also, there was a group of "specific" cortical cells.

About $^1/_4$ of the cells listed in table I responded to more than one type of stimulus. It is interesting to note that all these cells

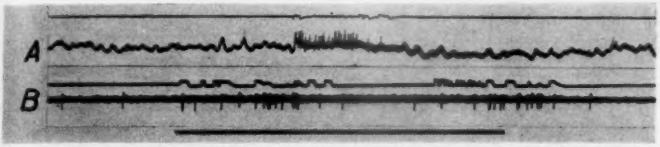

Fig. 1 A. Response of a cortical cell to touch on the ipsilateral tip of the tongue. Note: short latency and high frequency spike discharge on top of a slow negative potential. DC recording. B. Response of a cortical cell to pressure on the tongue. Note: gradual increase in frequency of the spontaneous discharge. This cell was also discharged by thermal stimuli. Amplifier time constant (TC): 5 msec. Time: 50 c. p. s. The irregularities of the top beams signal the application of the stimulus. Negativity in this and subsequent figures is recorded upwards.

could be discharged by impulses in afferent fibres of high conduction velocity *i. e.* they responded to touch or to stretch of the tongue as well as to thermal and/or gustatory stimuli. Their response shall now be considered somewhat more in detail.

Cortical Cells Responding to Thermal and Mechanical Stimuli.

The two most commonly observed combinations of effective stimuli were 1) cooling and "stretch" and 2) cooling and touch. Fig. 2 shows the impulse discharge recorded close to a cortical cell responding to cooling of the tip of the tongue and to touch within a narrow area (2×5 mm) along the very edge of the ipsilateral tip of the tongue. The cell showed a low spontaneous activity (< 1 imp./sec). Its response to touch, as seen in Fig. 2 A, consists of an irregular low frequency discharge with short latency to the first spike and a duration of 200 to 1,000 msec. Electrical stimulation within the receptive field on the tongue discharged this cell with a latency of 6—8 msec. Only one or two spikes were fired in response to each shock.

In Fig. 2 B, Ringer's solution of the tongue's temperature (29°) was poured onto the tongue as signalled on the top beam. Evidently the tactile stimulation caused by the flow of the solution was not strong enough to elicite a touch response. Cooling by applying Ringer's solution 12° to the tongue discharged the cell very effectively (C). Even a droplet of cold Ringer's solution on the tip of the tongue gave a good response. In contrast to the touch discharge the response to cooling showed a long latency. As previously reported (LANDGREN 1957) the latency is dependent on the intensity of the cooling (cf. Fig. 2 C and D). In C the latency is 150 msec., the duration of the response is 2 sec. and

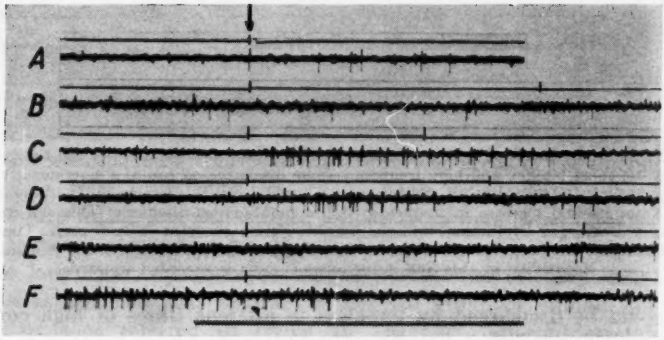

Fig. 2. Response of a cortical cell to touch and to cooling of the tip of the tongue. A. Touch, B. Ringer's solution 29°, C. Ringer's solution 12°, D. Water 23° after water 29°, E. Water 23° after water 7°, F. Water 40° after Ringer's solution 7°. The arrow and the vertical lines on the signal beam mark beginning and end of the application of the stimuli. Time: 50 c. p. s.

the mean frequency during the first second is 30 imp./sec. The response to cooling is thus much more conspicuous than that to touch.

Water 23° elicited the activity shown in D, when it was applied after previous flushing of the tongue with water 29°. On the other hand no response was obtained when the application followed upon a previous cooling with water 7° (E). When the tongue was warmed to 40° while the cortical cell was still responding vigorously to a previous cooling to 12° the response was cut short abruptly within 200—500 msec. after the application of warm water (F). Thus warming of the tongue did not discharge this cell.

A cortical cell, responding to cooling and to warming as well as to touch within a receptive field on the ipsilateral tip and edge of the tongue, is shown in Fig. 3. The spontaneous frequency of this cell was far below 1 imp./sec. In response to water 10° on the tongue the cell discharged a series of spikes with a latency of 300 msec. (A). The impulse frequency was 5—7 per sec. and the duration of the discharge about 2 sec. The cooling lasted 4 sec. and during the last two seconds of this period only one impulse was discharged per second. In the fifth and sixth second after the beginning of the stimulation another increase in the frequency (9 imp./sec.) was recorded, and, then, the cell returned to the control level of activity.

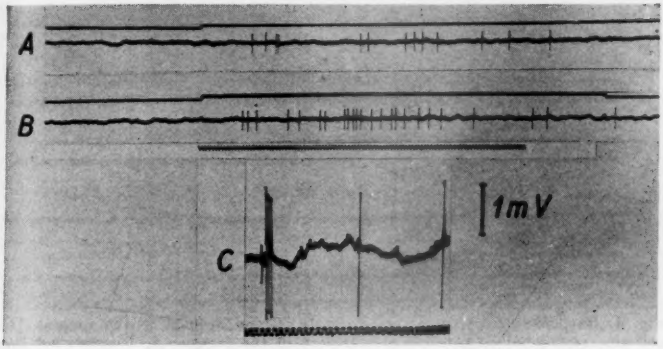

Fig. 3. Response of a cortical cell to cooling, warming and electrical stimulation of the tongue. This cell was also discharged by touch of the ipsilateral edge of the tongue. A. Water 10° and B. Water 43° applied to the tongue. Signal on top beam. Time: 50 c. p. s. C. Electrical stimulation of the ipsilateral edge of the tongue. Time: 10 msec. TC: 0.5 sec.

Warming of the tongue with water 43° during 3 seconds discharged the cell with a latency of 250 msec (Fig. 3 B). The response is similar to that of cooling, although the frequency is somewhat higher (12 imp./sec.). In this case also a delayed discharge was seen about 5 seconds after the beginning of the stimulation. The response to cooling and to warming were elicited even by a droplet of cold or warm water or Ringer's solution applied within the receptive field. No movements of the tongue were observed when the stimuli were applied. Squirting Ringer's solution or water of 30° onto the tongue did not discharge the cell, nor did taste solutions at the temperature of the tongue, cause a discharge. The response of this cell to touch with a glass rod was similar to that elicited by electrical stimulation.

Electrical stimulation of the tip of the tongue resulted in a burst of 3—4 spikes from this cell (Fig. 3 C). The positive-negative action potentials were riding on the negative focal potential. The latency of the first spike was 7 msec. In 50 % of the sweeps one or two spikes were fired with long latency (ca. 200 msec.), but in 50 % there was no after-discharge.

Cortical Cells Responding to Gustatory and Mechanical Stimuli.

As seen in table I taste and touch or taste and stretch were the effective stimuli of some cortical cells. Fig. 4 shows the activity of such a cell. It responded to rather hard pressure with

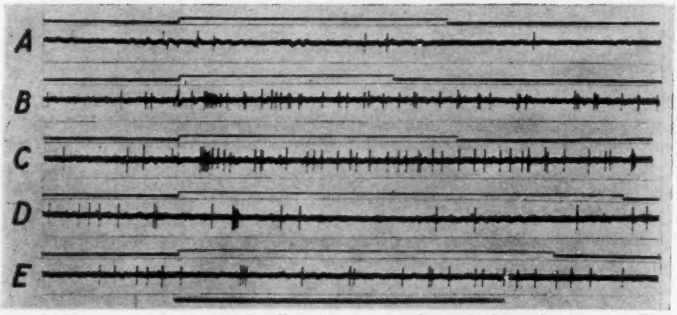

Fig. 4. Response of a cortical cell to taste stimuli applied to the tongue. This cell also responded to pressure and stretch of the tongue. A. Ringer's solution. B. 0.5 M NaCl, C. 0.3 M acetic acid, D. 0.01 M Quinine HCl, E. Distilled water. Signal on top beams marks the application of the solutions. Time: 50 c. p. s.

the glass rod on the middle third of the ipsilateral half of the tongue and when the tongue was stretched. The response to these stimuli was a moderate and gradual increase of the spontaneous frequency of the type shown in Fig. 1 B. The most effective stimuli to this cell were, however, salty and acid taste solutions. Touch, cooling and warming had no effect.

As seen in Fig. 4 A Ringer's solution did not discharge the cell. On the other hand 0.5 M NaCl and 0.3 M acetic acid in Ringer's solution (PH 3) gave rise to a long series of impulses initiated by a high frequency burst (Fig. 4 B and C). The latencies of the salt and acid responses were about 150 msec. and the duration exceeded 7 and 12 seconds respectively. The effects of 0.01 M Quinine solution and distilled water are shown in Fig. 4 D and E. The application of these solutions resulted in a burst of spikes of rather long latency (300—400 msec.), but no further significant changes of the spontaneous frequency were observed. If anything, the spontaneous frequency was decreased by these solutions.

Cortical Cells Responding to Thermal, Gustatory and Mechanical Stimuli.

Some cortical cells were discharged by thermal, gustatory and mechanical stimuli. Fig. 5 shows the behaviour of such a cell. It responded when the tongue was stretched (B) and when the cat made attempts to swallow. A short burst of spikes appeared when Ringer's solution of the tongue's temperature (30°) was applied (A). Distilled water of the same temperature gave a

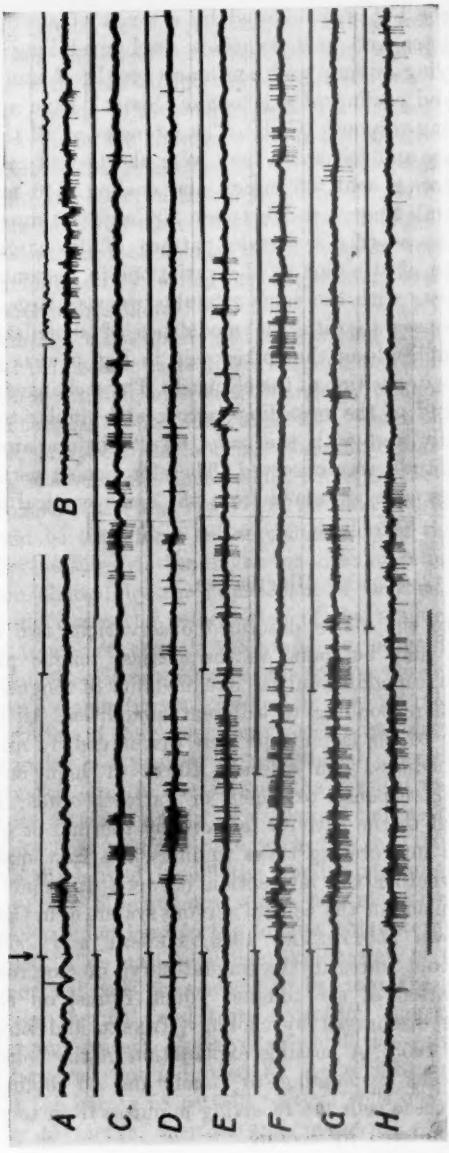

Fig. 5. Response of a cortical cell to pressure and stretch of the tongue, to taste stimuli and to warming and cooling of the tongue. A. Ringer's solution 30°, B. Stretch of the tongue, C. Water 30°, D. 0.5 M NaCl, E. 0.01 M Quinine HCl, F. 0.3 M acetic acid, G. Water 42°, H. Water 17°. Signals on top beams mark the application of the stimuli. Time: 50 c. p. s.

similar response but was followed by a series of after-discharge (C). Salty, bitter and acid solutions discharged long series of spikes of varying latency and configuration (D, E and F). Also to warming and cooling of the tongue this cell gave a vigorous and long lasting response (G, H). The latencies of all these reactions were long and variable. They were shortest in response to stretch (85 msec.), acid (85 msec.) and cooling (140 msec.). To the other stimuli they varied between 200 and 600 msec. It was not possible to ascribe a certain pattern of discharge to one particular type of stimulus as the variations in response to two consecutive tests with the same stimulus were as large as those between responses to different modalities of stimulation. The changes in amplitude of the spikes seen in Fig. 5 were probably due to changes in position of the electrode. These changes occurred towards the end of the recording period, and similar tests were done repeatedly and with the same results before any change in spike amplitude was observed. Therefore, most certainly all these responses were obtained from the same cortical cell.

Discussion.

According to the above described observations two different types of cells may be found in the cortical tongue projection area, *i. e.* those responding only to one modality of tongue stimulation and those responding to different modalities. All observed cells belonging to the latter type were discharged by mechanical stimulation mediated via afferent fibres of high conduction velocity. Their response to touch or "stretch" was, however, weak compared to the activity induced by thermal or gustatory stimuli. When interpreting these findings, the first question to be asked is whether the interaction between different types of stimuli takes place in the central nervous system or in the periphery. It is known (Zotterman 1936, Hensel and Zotterman 1951 a) that cold fibres in the lingual nerve do not respond to tactile stimulation of the tongue. Touch fibres, on the other hand, may be discharged by cooling (Hensel and Zotterman 1951 b, Dodt 1953). A possible explanation of the behaviour of the cortical cells responding to touch and to cooling would, then, be that these cells are receiving impulses from touch fibres which are stimulated by cooling.

The long latency of the response to cooling compared to that recorded from the same cell when touching the tongue may be compatible with such an interpretation. However, a touch fibre, excited by cooling of the tongue, responds with a short series of spikes at a moderate frequency compared to the long lasting and frequent discharge elicited by continuous tactile stimulation (cf. HENSEL and ZOTTERMAN 1951 b Fig. 1). In contrast, these cortical cells are fired more effectively by cooling than by touch. It is, therefore, improbable that the activity induced in a cortical cell by touching and by cooling the tongue could be due to stimulation of touch fibres alone.

Touch fibres are not discharged by warming or by taste stimuli, nor do warm fibres or taste fibres respond to touch. Nonspecific stimulation of touch afferents, therefore, cannot explain the activity of cortical "touch and warm" and "touch and taste" cells.

There is, however, still another possibility that the impulses elicited by the various stimuli could travel in the same afferent fibres. Because of the long latency of the cold and taste responses there would be time for a reflex contraction of the tongue and masticatory muscles via the linguo-mandibular reflex. This reflex contraction elicited by the application of thermal or gustatory stimuli could cause the deformation of the tongue necessary to stimulate touch or stretch receptors. In order to avoid this complication the efferent nerves of the tongue were cut bilaterally. Cortical cells responding to swallowing, jaw movements and not localized stretch of the tongue may receive impulses from stretch receptors in the masticatory or pharyngeal muscles, which were not denervated, and in these cases the cortical response to different types of stimuli may be explained on the basis of reflex contraction. This was the case with the cell shown in Fig. 5 which was discharged by all the applied stimuli except touch. In most of the observations where the receptive field of the cortical cell was localised to the tongue, this interpretation could be discarded. The most plausible explanation of the behaviour of these cortical cells thus seems to be, that the afferent paths from tactile, thermal, and taste receptors converge within the central nervous system. The level at which the convergence takes place is, however, not indicated by the present experiments. It may be in the cortex as certain cortical cells obviously respond specifically to tactile, thermal and gustatory stimuli. Many of these specific cortical cells were shown to respond with a short

latency (COHEN, LANDGREN, STRÖM and ZOTTERMAN 1957, LANDGREN 1957). The cortical cells discharged by more than one type of stimulus have generally responded after a relatively long latency. They may, therefore, be activated secondarily by specific primary cortical cells.

It is tempting to assume that cortical cells showing an increased activity in response to stimulation of the tongue are engaged in the perception of tongue sensations. If this assumption is true, an interaction between on the one hand tactile and thermal and on the other tactile and gustatory stimuli should play a role in eliciting thermal and gustatory sensations respectively. This view is supported by the findings of HARRIS (1952) who showed that destruction of the trigeminal paths in humans caused a temporary abolition of taste even though the chorda tympani and glossopharyngeal pathways for taste fibres were intact. A reasonable working hypothesis concerning a central interaction between tactile, thermal and taste impulses would be that the first mentioned exert a general facilitatory action on the cortical cells receiving impulses of the two latter modalities. Some necessary conditions for such an hypothesis certainly exist. Thus touch and "stretch" are conducted centrally in the fastest of the tongue afferents. Impulses in these fibres elicit the large initial component of the evoked potential in the tongue projection area (COHEN, LANDGREN, STRÖM and ZOTTERMAN 1957). Cortical cells responding to touch were often found in the neighbourhood of taste and cold cells sometimes at a distance of only 50 μ within the same microelectrode track. As the mechanoreceptors, thermoreceptors and taste receptors of the tongue are stimulated simultaneously under physiological conditions the slowly conducted thermo and taste impulses will arrive in the cortex in the wake of a cortical response to mechanical stimulation. The initial cortical response to tactile stimulation is, further, elicited within a closely mixed pool of cortical cells responding "specifically" or "nonspecifically" to the different types of impulses from the tongue.

Summary.

Using the extracellular microelectrode technique 325 cortical cells in the tongue projection area were studied and tested by the application of mechanical, thermal, gustatory or electrical

stimuli to the tongue. Out of this group of tested cells 101 cells responded to tongue stimulation. The majority of these responding cells were discharged by one type of stimulus only, but 27 cells were activated by fibres subserving different sensory modalities. The usually observed combinations of effective stimuli were cooling and touch or cooling and "stretch", but cortical cells responding to taste and touch or taste and "stretch" were also found. In some cells even more complicated combinations of effective stimuli were observed.

All cortical cells of the type responding to more than one modality were discharged via rapidly conducting afferent paths from touch or stretch receptors in the tongue. Thermal and gustatory stimuli on the other hand, activated these same cells more efficiently but with a longer latency (100—500 msec.) than did mechanical stimulation.

It was concluded that the observed convergence of tactile, thermal and gustatory impulses occurred in the central nervous system.

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Primary Afferent Collaterals and Spinal Relays of the Dorsal and Ventral Spino-Cerebellar Tracts.

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In recent papers the spino-cerebellar tracts have been analysed with electrophysiological methods. The dorsal spino-cerebellar tract (DSCT) has been shown to be activated from ipsilateral muscle spindle and Golgi tendon organ afferents (LAPORTE, LUNDBERG and OSCARSSON 1956 a and b, LAPORTE and LUNDBERG 1956, LUNDBERG and OSCARSSON 1956). The results indicated that the DSCT should be subdivided into two parts, one forwarding messages from muscle spindles and one messages from Golgi tendon organs. The ventral spino-cerebellar tract (VSCT) has been shown to be monosynaptically activated from contralateral Golgi tendon organ afferents (OSCARSSON 1956).

The cells of origin of the DSCT are well known and constitute Clarke's column. This column of cells extends from the upper thoracic segments to the third or fourth lumbar segment in the cat (REXED 1954). The VSCT cells, on the other hand, are not identified though there is some histological evidence for a more caudal extension of the column of VSCT cells than of Clarke's column (cf. YOSS 1953).

The finding of two functionally different pathways in the DSCT made it of interest to investigate if these followed the same anatomical course. In this paper will be shown that the collaterals of the primary afferents from Golgi tendon organs take a course different from those from muscle spindles but that both types

of afferents make synaptic contacts with DSCT cells located at the same segmental level. It will further be shown that the synaptic relay of the VSCT is at a lower segmental level, and that the collaterals from Golgi tendon organ afferents going to the VSCT neurons take a similar course as those going to the DSCT cells.

Methods.

The experiments were performed on cats under nembutal anaesthesia (40 mg/kg intraperitoneally). The spinal cord was exposed in the upper thoracic region for three to four cm and also from the Th XII segment down to the first sacral segment. In the upper thoracic region the spinal cord was transected at the cranial end of the exposure and after dissecting away the dorsal funiculi the rest of the cord was divided into two halves which were mounted on electrodes for recording from the spino-cerebellar tracts (cf. LAPORTE et al. 1956 a, OSCARSSON 1956). The hindlimbs were denervated and the hamstring nerve on one side dissected for stimulation.

Transections of the dorsal funiculi in order to sever the primary afferents were performed under visual control through a binocular dissecting microscope. Before cutting the funiculi a narrow band of pia was removed in order to diminish tearing of the white matter. Longitudinal sections were performed similarly but without previous removing of the pia. The lesions were afterwards controlled under microscope. The rectal temperature was held within 36—38.5° C.

Results.

It is well known that the primary afferents making synaptic contacts with the neurons of Clarke's column ascend in the dorsal funiculus and reach the neurons via collaterals which more or less perpendicular to the axis of the spinal cord pass down to the column (cf. LLOYD and MCINTYRE 1950, SZENTAGOTHAI and ALBERT 1955). The afferents to the VSCT neurons also belong to large fibres known to ascend in the dorsal funiculi (LUNDBERG and OSCARSSON 1956, OSCARSSON 1956), and it seems very likely that also these fibres reach their destination via collaterals similarly arranged. With the intention of severing the afferents before they give off the collaterals and thereby interrupting the afferent pathway to the tract neurons the dorsal funiculi were transected at successively more caudal levels. At the same time the mass discharges in the ventral and dorsal spino-cerebellar tracts were recorded on stimulation of the hamstring nerve (or in a few cases only part of it: the biceps p. + semitendinosus nerve). The same

nerve was used in all the experiments as to make comparison possible. If the collaterals to the cell pools of the tracts were given off at different levels the discharge in the tract with the more cranially located collaterals should decrease and disappear before the discharge in the other tract. During the experiments, however, the anatomical organization of the collaterals was found to be more complicated than was originally assumed. The functionally different types of afferents (those from muscle spindles and those from Golgi tendon organs) to DSCT neurons were found to reach their destination along different courses which could be explored by use of the separation of the group I volley in its two components, which correspond to the coarse muscle spindle afferents and the Golgi tendon organ afferents respectively (BRADLEY and ECCLES 1953, ECCLES et al. 1957, LAPORTE and BESSOU 1957). These components will here be denoted as the Ia and Ib volleys (cf. LUNDBERG and OSCARSSON 1956).

Fig. 1 illustrates such an experiment. The upper row of records was taken on stimulation slightly supramaximal to group Ia as judged from triphasic dorsal root recordings (not shown in the figure). Only a very small part of the Ib volley was included and the discharge in the DSCT (upper beam) is mostly due to the Ia contribution of the mass discharge. The small Ib volley results in a just visible discharge in the VSCT (lower beam). Records 4—6 show the discharges on slightly supramaximal group II stimulation. The first spike of the DSCT discharge is in this case due to the combined Ia and Ib contributions and is followed by a second spike seen as a small hump in record 4 and mostly due to excitatory action from group II fibres (LAPORTE et al. 1956 b). The left records were taken before any transection of the dorsal funiculi. It is evident from records 1 and 4 that both Ia and Ib contributes considerably to the DSCT discharge. As usually the VSCT discharge is somewhat smaller than the DSCT one. Records 2 and 5 were taken after transection of the dorsal funiculi in the upper part of the L IV segment and show a great reduction of the Ia contribution to the DSCT discharge. As this discharge is partly caused by the rudimentary Ib volley included, the Ia contribution is even somewhat smaller than shown in record 2. On supramaximal Ib stimulation the DSCT discharge increases considerably showing that the Ib contribution is virtually undiminished. The latency for the DSCT discharge has increased

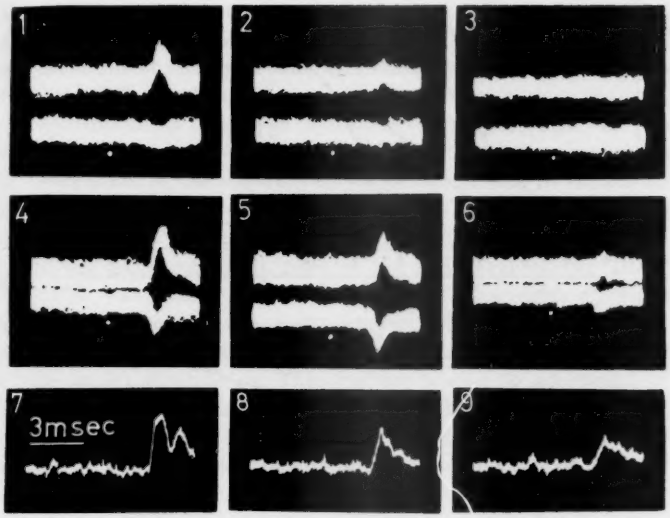

Fig. 1. The decrease of the discharges in the spino-cerebellar tracts after transections of the dorsal funiculi.

Superposed recordings from dissected spinal halves (except dorsal funiculi) to show the DSCT (upper beam in records 1—6) and the VSCT discharge (lower beam) on stimulation of the hamstring nerve. Dots mark stimulus artefacts. Records 1—3 with stimulation slightly supramaximal to group Ia giving a Ia volley + a very small Ib volley as judged from triphasic dorsal root recordings (not shown in the figure). The small Ib volley causes a just visible discharge in the VSCT. Records 4—6 with slightly supramaximal group II stimulation. Note increased latency for the DSCT discharge in records 5 and 6. Records 7—9 with the same stimulus strength as in records 4—6 but with single sweeps in order to show the second spike of the DSCT discharge. Left records taken previous to any transection of the dorsal funiculi. Middle vertical row of records after transection in the cranial part and record 9 after transection in the caudal part of the L IV segment. Records 3 and 6 after transection in the caudal part of the L V segment. The same sweep speed and amplification in all records.

slightly due to the slower conduction velocity of the Ib volley. The VSCT discharge is unreduced in record 5.

Records 3 and 6 show the discharges after a transection in the lower part of the L V segment. This resulted in an approximately parallel decrease of both discharges.

The successive decrease of the mass discharge in the two spino-cerebellar tracts after each transection is shown by the curves in Fig. 2 A. The continuous lines indicate the decrease of the discharge on maximal group I stimulation. The discharge in the DSCT begins to diminish at the upper part of the L III segment and the discharge in the VSCT at the upper part of the L IV

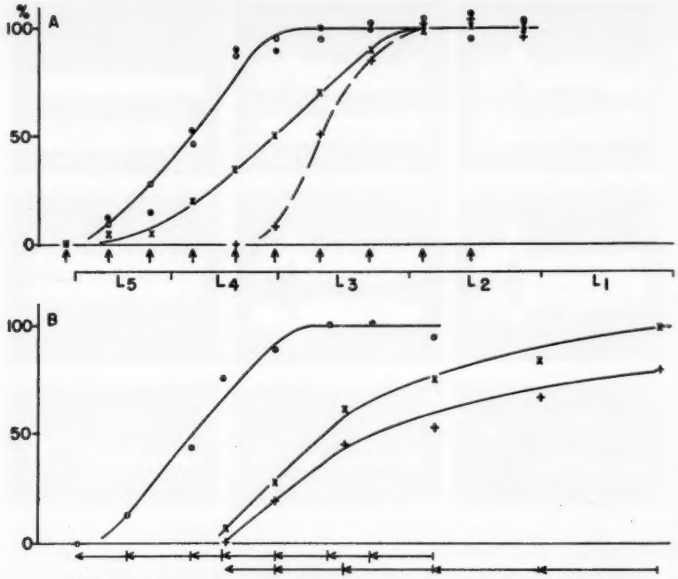

Fig. 2 A. The decrease of the amplitude of the discharges in the DSCT and VSCT on stimulation of the hamstring nerve after transections of the dorsal funiculi (marked as arrows on a scale representing spinal segments). Continuous lines represent the discharges in the DSCT (crosses) and the VSCT (open circles) on stimulation slightly supramaximal to group II. Broken line represents the discharge in the DSCT (pluses) on stimulation of group Ia. The Ib contribution to the DSCT discharge (filled circles) was obtained by subtracting the amplitude of the maximal DSCT discharge with the amplitude of the discharge due to the Ia contribution. Each plotting represents mean of ten recordings. B. Curves from two experiments. The decrease of the mass discharge in the VSCT (circles) after longitudinal median sections of the spinal cord (indicated by upper row of arrows) on supramaximal stimulation of the hamstring nerve. The decrease of the amplitude of the mass discharge in the DSCT on hamstring nerve stimulation after longitudinal sections as described in the text (indicated by lower row of arrows). Crosses represent the discharge due to a supramaximal group I volley and pluses the discharge due to the Ia contribution (in per cent of the total original discharge). Each plotting represents mean of ten recordings.

segment. The decrease of the DSCT discharge is less steep than that of the VSCT one resulting in a complete disappearance of both discharges after transection at the same level (the lower part of the L V segment). The interrupted line shows the decrease of the Ia contribution to the DSCT discharge and the filled circles the decrease of the Ib contribution. The latter decrease

is closely concomitant with that of the VSCT discharge. This indicates that the Ib collaterals to both DSCT and VSCT neurons are given off at the same level and caudally to the Ia collaterals to the DSCT neurons.

Experiments of the type described were performed on five cats, in three of which group Ia and Ib separation permitted determination of the different levels of the Ia and Ib collaterals to the DSCT. In all the experiments the results were in good conformity with those illustrated in Figs. 1 and 2 A. In all the cases the possibility that part of the reduction of the DSCT discharge was due to damage of Flechsig's fasciculus was eliminated by checking the early fraction of the mass discharge appearing on ipsilateral skin nerve (the superficial peroneal nerve at the ancle) stimulation. This fraction is due to activation of fibres within Flechsig's fasciculus and is not reduced by transections before these are performed caudally to those abolishing the VSCT discharge (Oscarsson 1957). No reduction or at most a decrease of 5—10 per cent was found indicating that the reduction of the DSCT discharge was only insignificantly due to injury of the tract fibres.

Lundberg and Oscarsson (1956) discussed the possibility of convergence onto DSCT neurons from Ia and Ib fibres but gave evidence indicating that most of the DSCT neurons were predominantly excited from either Ia or Ib primary afferents. The possibility that neurons activated mainly from Ib fibres were located caudally of those activated mainly from Ia afferents was tested by following the reduction of the mass discharge in the DSCT on longitudinal sectioning of the spinal cord in order to interrupt the DSCT axons as they entered Flechsig's fasciculus. The sections were performed in a plane going through the lateral border of the ipsilateral dorsal funiculus and the anterior median fissure. The sectioning was begun at the upper part of the L I segment and recordings were made after each 5—10 mm's sectioning in caudal direction. If the two groups of DSCT neurons were located at different levels the contributions from Ia and Ib should decrease separately in a manner similar to that in the experiments with transections of the dorsal funiculi. Three experiments were made and gave similar results. The plotting of the mass discharge in Fig. 2 B shows that the discharges due to Ia and Ib diminished concomitantly and at the level corresponding to the Ia collaterals. That suggests that the Ib collaterals after

entering the gray matter ascend and make synaptic contacts with DSCT neurons at the same level as those innervated by the Ia collaterals. In good conformity with this SZENTAGOTHAI and ALBERT (1955) reported that some dorsal root afferents after entering Clarke's column ascend for a certain distance before terminating on DSCT neurons. This may also explain why part of the DSCT discharge disappeared already at lesions in the L I segment as is shown by the curve.

The second spike in the mass discharge of Flechsig's tract, mainly due to stimulation of group II fibres, was small in the superposed records (cf. record 4, Fig. 1). These were obtained with a rather high stimulation frequency in order to obtain good records. With a lower frequency the second spike appeared more distinctly and from single records it was possible to appreciate the level of its disappearance. This is shown in records 7—9 in Fig. 1. Record 7 gives the discharge before any transection. Most of the second spike has disappeared in record 8 (which corresponds to records 2 and 5) and in the last record it has disappeared completely. The decrease of this potential was concomitant with the decrease of the Ia contribution indicating that the collaterals from both types of muscle spindle afferents are given off at the same level.

In order to ascertain if the VSCT neurons were located at the level corresponding to the Ib collaterals the cord was longitudinally sectioned in the midline with similar technique as for sectioning the DSCT axons. The result is shown in Fig. 2 B (circles) and suggests that the VSCT neurons are located caudally of the Clarke's column cells and at the same level as the Ib fibres give off their collaterals. This conclusion is valid if it is the axons of the secondary neurons (not the primary afferents), the VSCT neurons, that cross to the other side as is generally agreed (JANSEN and BRODAL 1954).

An attempt was made to confirm the results showing different levels of branching of the primary afferents by recording the volley from the dorsal funiculi. LLOYD and MCINTYRE (1950) showed that the conduction velocity on muscle nerve stimulation was reduced at two (in some cases at three) levels. The first reduction corresponded to the level where the collaterals to motoneurons were given off and the second to the level of the collaterals to Clarke's cells. By plotting the Ia and Ib volleys separately the results by LLOYD and MCINTYRE were confirmed but attempts to determine the level of the second decrease of the velocity of the Ib volley were not successful as this volley was temporally dispersed at the upper lumbar segments (three experiments).

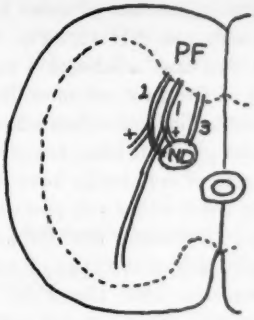

Fig. 3. Schematic drawing illustrating the development of collaterals from primary afferents to Clarke's column in human embryos according to Hogg (1944). PF, dorsal funiculus. ND, Clarke's column. See the text.

Discussion.

Afferents from Golgi tendon organs and both types of afferents from muscle spindles converge onto DSCT neurons whereas the VSCT neurons are monosynaptically activated mainly or exclusively from Golgi tendon organ afferents. In this paper is shown that the Ib collaterals (hamstring nerve) take an anatomical course which is separate from that of the collaterals of the muscle spindle afferents. The latter are given off at the caudal end of Clarke's column (cf. Lloyd and McIntyre 1950, Rexed 1954) whereas the Ib collaterals both to the VSCT and DSCT neurons are given off at a more caudal level which corresponds to the level of the VSCT neurons. It seems not unreasonable to presume that the Ib collaterals for the two tracts are identical initially. That should explain the unique course to the DSCT neurons taken by the Ib afferents, the collaterals of which after branching and sending secondary collaterals to the VSCT neurons, should ascend within the grey matter to make synaptic contacts with the DSCT neurons located at a more cranial level.

It is of interest to relate these findings with those of Hogg (1944) on the development of afferent collaterals to Clarke's column in human foetuses. Fig. 3 summarizes Hogg's results. From the dorsal funiculus (PF) three sets of collaterals grow towards Clarke's column (ND) at different times during the development. The first set of afferents to appear in the gray matter grows down to the periphery of the anterior gray column

(1) and somewhat later a new set of more lateral fibres take an arcuate course to reach the ND (2). The last bundle appears medially of the first two and is labeled 3 in the figure. Slightly later, branches (1+) of the first set of collaterals grow medially to come in contact with Clarke's column and at about the same time also the second set gives off branches (2+) which are directed laterally. The three sets of collaterals have thus different courses and two of them also reach other cell pools in the cord. LAPORTE et al. (1956 b, c) and LUNDBERG and OSCARSSON (1956) showed that three functionally different types of muscle afferents make monosynaptic connections with the DSCT neurons. It seems highly probable that the three sets of collaterals described by HOGG represent these three types of afferents and thus correspond to the two types of afferents from muscle spindles and the afferents from Golgi tendon organs. On the other hand, the VSCT neurons appear to be monosynaptically innervated only by Ib afferents (OSCARSSON 1956). HOGG actually suggested that the branches of bundle 2 should represent connections to the VSCT, but any further discussion of this problem would be premature since we do not known the location of the VSCT cells.

Summary.

The experiments were performed on cats under nembutal anaesthesia.

Successively more caudal midline sections of the spinal cord abolished the ventral spino-cerebellar tract (VSCT) discharge evoked on hamstring nerve stimulation, only when the sections were performed at the lower part of the L V segment. This indicates that the column of VSCT cells extends to a level caudally of Clarke's column.

The mass discharges in the dorsal and ventral spino-cerebellar tracts (DSCT and VSCT) were simultaneously recorded on stimulation of the hamstring nerve. The decrease of the discharges after sections of the dorsal funiculi at different segmental levels showed that the collaterals from Golgi tendon organ afferents to both DSCT and VSCT neurons are given off at the same segmental level. This level corresponds to the location of the VSCT neurons, caudally of Clarke's column. Both types of muscle spindle afferents ascend in the dorsal funiculi, and give off collaterals to the DSCT neurons at a more cranial level, which corresponds to the caudal-

most part of Clarke's column. It is suggested that the collaterals of the Golgi tendon organ afferents are common for the two tracts and that these collaterals branch to give secondary collaterals to the VSCT neurons and other secondary collaterals which ascend within the gray matter to terminate on the DSCT neurons.

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On The Specificity of Pancreatic Lipase.

By

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The mechanism of pancreatic lipolysis of glycerides containing ordinary straight-chain fatty acids has earlier been studied in this institute (Borgström 1952 b, 1954 a, b). *In vitro* and *in vivo* experiments showed that during the hydrolysis of triglycerides by pancreatic lipase an exchange of glyceride fatty acids and unesterified fatty acids takes place (*cf.* Borgström 1952 b).

Recently it was found that 2,2-dimethylstearic acid did not take part in the formation of ester bonds during hydrolysis of olive oil with pancreatic lipase (Bergström, Borgström, Tryding, and Westöö 1954). Likewise, biosynthesized glyceride ester bonds with this branched-chain fatty acid were found to be virtually resistent to the action of the lipase *in vitro*. Nor were these glycerides hydrolyzed in the intestinal lumina of rats during the active absorption phase (Blomstrand, Tryding and Westöö 1956).

In this investigation the specificity of pancreatic lipase has been studied with the aid of different methyl-substituted fatty acids. Olive oil containing small amounts of labelled, methyl-branched acids has been hydrolyzed by pancreatic lipase *in vitro*. In addition, glycerides containing the labelled fatty acids have been digested by the lipase *in vitro*.

Experimental.

The enzyme preparation used was lyophilized, pure, rat pancreatic juice (Borgström 1954 a). Three mg of the dry enzyme powder (kindly supplied by Dr. B. Borgström) were taken for each 100 mg

of the substrate. The main constituent of the substrates was a commercial preparation of olive oil. The acids included in the investigation were:

^{14}C-labelled acids:

1. [1-^{14}C]Palmitic acid (0.57 μC/mg, i.e., $4.75 \cdot 10^5$ cpm/mg when assayed in an "infinitely thin" layer in a gas flow counter) prepared according to Bergström, Borgström and Rottenberg (1952).
2. [1-^{14}C]Oleic acid (0.14 μC/mg) prepared as described by Bergström, Pääbo and Rottenberg (1952).

These two acids were kindly supplied by Professor S. Bergström.

3. 2,2-Dimethyl[1-^{14}C]nonadecanoic acid (0.15 μC/mg) was prepared by Tryding and Westöö (1956).
4. 2-Methyl[1-^{14}C]stearic acid (0.13 μC/mg) was prepared by Tryding and Westöö (1957 a).
5. 2,2,17,17-Tetramethyl[1-^{14}C]stearic acid (0.18 μC/mg) was prepared by Tryding and Westöö (1957 b).

Tritium-labelled acids:

6. 5,5-Dimethylhexanoic acid with a specific activity of $1 \cdot 10^5$ cpm/mg. This acid was synthesized, tritium-labelled and chromatographed by Tryding, Lindstedt and Westöö (to be published).
7. 15,15-Dimethylpalmitic acid (s.a.: $1 \cdot 10^8$ cpm/mg). The synthesis was made by Tryding and Westöö (to be published) while the tritium-labelling (platinum-catalyzed exchange in tritiated water-ethanol/sodium hydroxide) was performed by Dr. S. Lindstedt.
8. (+)-3D-Methylstearic acid (s.a.: $21 \cdot 10^6$ cpm/mg).
9. 3DL-Methylstearic acid (s.a.: $25 \cdot 10^6$ cpm/mg).
10. (+)-4D-Methyltetracosanoic acid (s.a.: $12 \cdot 10^6$ cpm/mg).
11. *n*-Hexacosanoic acid (s.a.: $18 \cdot 10^6$ cpm/mg).

The acids 8—11 have kindly been placed at my disposal by Professor E. Stenhagen. For the methods of synthesis see Ställberg-Stenhagen (1951).

Crystals (20 mg) of the fatty acids 8—11 were placed in an atmosphere of 89 % tritium gas (1 Curie) at room temperature for 4 days (*cf.* Wilzbach 1957). (The tritium-labelling was carried out by Dr. S. Lindstedt).

Each of the labelled acids 8—11 was subjected to reversed-phase partition chromatography with liquid paraffin as the stationary phase and 70 % acetone: water (v,v) as the moving phase (Howard and Martin 1950). On a 4.5 g column of hydrophobic kieselguhr the active peak of the 3-methylstearic acids appeared at about 55 ml. Less than 10 % of the activity moved with the front, while less than 0.1 % remained on the column. The long-chain C_{25}- and C_{26}-acids both stayed on the column while the more polar impurities were eluted with the moving phase. Before use, also the acids 1—5 and 7 were purified by partition chromatography according to Howard and Martin.

General procedure.

Olive oil (100 mg) containing the labelled free acids (1 mg) was incubated with the enzyme preparation (3 mg) in a Tris-buffer (8 ml) with a pH of 8.0. Generally 0.05 M Tris-HCl buffer was used but in a series of experiments (Table 2) it was replaced with 0.2 M Tris-HCl. The tubes were rotated at about 40 r.p.m. in a water bath (37°) for 18 or 9 hours. After acidification with 6 N hydrochloric acid the fat was extracted three times with two volumes of ether. The combined ether extracts were washed with small portions of water and dried with anhydrous sulfate. After weighing the fat was subjected to chromatography on 20 ml columns of Amberlite IRA-400 (Borgström 1952 a).

In connection with studies on the metabolism of branched-chain fatty acids we have made several tests on the ability of Amberlite IRA-400 to separate different free, branched, long-chain fatty acids from their glycerides. The results of these control experiments were in complete accordance with those obtained by Borgström (1952 a, b) for ordinary straight-chain fatty acids.

When tritium-labelled *n*-hexacosanoic acid (0.3 mg, i.e., $5.4 \cdot 10^6$ cpm), olive oil (60 mg) and free, unlabelled stearic acid (40 mg) were chromatographed together on a 20 ml column of Amberlite IRA-400, 1.7 % of the activity passed through, the rest being taken up be the ion-exchanger resin.

In a series of experiments 2-methyl[1-^{14}C]stearic acid, 2,2,17,17-tetramethyl[1-^{14}C]stearic acid and tritium-labelled 15,15-dimethylpalmitic acid were fed to rats with thoracic duct lymph fistulas. The lymph fat was extracted and the triglycerides isolated (*cf.* Borgström and Tryding 1956). The biosynthesized glycerides (100 mg) were incubated at 37° for 18 hours with pancreatic lipase (3 mg) in a Tris-buffer pH 8.0 (8 ml). The fat mixture was extracted and fractionated as described above.

The neutral fat fractions were saponified in alcoholic potassium hydroxide and the acids were extracted with ether from the acidified water phase. The specific activities of the glyceride fatty acids and the free fatty acids were determined after direct plating on aluminium planchets. 0.1 mg of the tritium-labelled material, or 1.0 mg of the ^{14}C-labelled material was plated on each planchet (area: 7 sq. cm). They were counted in a gas flow counter ("Tracerlab", with a background effect of 30 cpm).

Results.

Table 1 and 2 summarize the results of the incorporation of free fatty acids into glyceride ester bonds during lipase hydrolysis of olive oil *in vitro*. The straight-chain palmitic acid, oleic acid and hexacosanoic acid as well as the branched-chain 4-methyltetracosanoic acid, 15,15-dimethylpalmitic acid and 5,5-dimethylcaproic acid were all relatively well incorporated into the glycerides. The fatty acids of another group, including the α- or β-substituted acids, were in-

Table 1.

Incorporation of different, labelled fatty acids into glycerides during hydrolysis in vitro by rat pancreatic lipase (0.05 M Tris buffer pH 8.0 at 37° for 18 hours).

Substrate: Olive oil (100 mg) containing 1 mg of free:	Total fat recovered mg	Per cent of hydrolysis according to weight	Specific activity of neutral fat fatty acids cpm/mg	Specific activity of free fatty acids cpm/mg	Specific activity of neutral fat fatty acids in per cent of the s.a. of free fatty acids
5,5-dimethylcaproic acid-T	93.5	48	490	1,400[1]	35
palmitic acid-^{14}C	94.9	41	836	3,340	25
oleic acid-^{14}C	90.5	47	530	1,400	38
15,15-dimethylpalmitic acid-T	94.4	38	4,580	13,670	34
2-methylstearic acid-^{14}C	92.2	40	30	3,025	1
3DL-methylstearic acid-T	95.5	42	2,700	97,500	3
(+)-3D-methylstearic acid-T	93.7	35	2,670	84.950	3
2,2-dimethylnonadecanoic acid-^{14}C	94.6	40	14	3,080	0.5
2,2,17,17- tetramethylstearic acid-^{14}C	90.4	40	26	2,180	1
(+)-4D-methyltetracosanoic acid-T	90.8	39	34,900	117,500	30
n-hexacosanoic acid-T	90.6	35	46,300	164,500	28

[1] This figure has been calculated from the s.a. of the total fat fatty acids and the s.a. of the neutral fat fatty acids.

Table 2.

Incorporation of labelled fatty acids into glycerides during hydrolysis in vitro by rat pancreatic lipase (0.2 M Tris buffer pH 8.0 at 37° for 9 hours).

Substrate: Olive oil (100 mg) containing 1 mg of free:	Total fat recovered mg	Per cent of hydrolysis according to weight	Specific activity of neutral fat fatty acids cpm/mg	Specific activity of free fatty acids cpm/mg	Specific activity of neutral fat fatty acids in per cent of the s.a. of free fatty acids
palmitic acid-^{14}C	92.6	67	620	1,350	46
2-methylstearic acid-^{14}C	96.5	69	48	2,325	2
3DL-methylstearic acid-T	91.3	68	5,000	82,500	6
	90.2	62	3,260	80,100	4
(+)-3D-methylstearic acid-T	95.6	65	7,450	75,600	10
	92.9	62	7,250	72,000	10
2,2-dimethylnonadecanoic acid-^{14}C	93.2	70	40	2,810	1
(+)-4D-methyltetracosanoic acid-^{14}C	94.0	64	45,600	86,000	53

Table 3.

In vitro hydrolysis by rat pancreatic lipase of biosynthesized glycerides containing labelled fatty acids (Tris buffer pH 8.0 at 37° for 18 hours).

Substrate: Biosynthesized glycerides (100 mg) containing labelled	Total fat recovered mg	Per cent of hydrolysis according to weight	Specific activity of neutral fat fatty acids cpm/mg	Specific activity of free fatty acids cpm/mg	Specific activity of the free fatty acids in per cent of the s.a. of neutral fat fatty acids
2-methylstearic acid-^{14}C ..	95.0	39	2,283	48	2
2-methylstearic acid-^{14}C ..	89.7	44	4,575	156	3
15,15-dimethylpalmitic acid-T	92.7	38	3,940	2,320	59
2,2,17,17-tetramethylstearic acid-^{14}C.....	93.1	41	1,805	28	2

corporated into ester bonds only to a small extent. In fact some of the activities of the neutral fat fatty acids were so small that they may have been insignificant. The results of the hydrolysis of biosynthesized triglycerides by pancreatic lipase are shown in Table 3. It is seen that the glycerides of 15,15-dimethylpalmitic acid were readily hydrolyzed, while only small amounts, if any, of the 2-methylstearic acid and 2,2,17,17-tetramethylstearic acid were set free by the action of the lipase.

The figures for the incorporation were low when the 0.05 M buffer was used (Table 1) which most probable was due to the fact that the pH changed from 8.0 to 7.6 during the hydrolysis. In another series of experiments a 0.2 M Tris-buffer was used with a pH-change from 8.0 to 7.85. The incorporation of the labelled acids was now higher (Table 2).

Discussion.

From Tables 1 and 2 it is seen that the fatty acids with methyl branches at the α- or β-carbon atom, at most to a small extent took part in the formation of new ester bonds during pancreatic lipase hydrolysis of olive oil. In accordance with these results it has also been found that glyceride ester bonds with α-methyl-substituted fatty acids were not split when incubated with pancreatic lipase, while glycerides with the 15,15-dimethyl-branched acid were readily hydrolyzed under the same conditions (*cf.* Table 3).

Our results are in agreement with the earlier findings of Balls, Matlack and Tucker (1937) that in comparison to

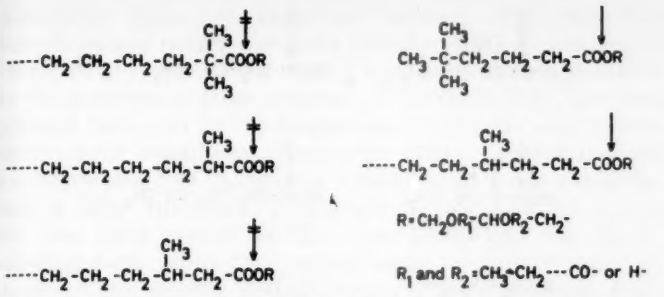

Fig. 1. Scheme of synthesis of glyceride ester bonds with different methyl-branched fatty acids by rat pancreatic lipase. ‡ = hindered enzymic synthesis. ↓ = unhindered enzymic synthesis.

glycerides with straight-chain fatty acids, tri-*iso*-valerin (tri-β-methylbutyrin) is poorly hydrolyzed through the action of pancreatic lipase.

The methyl groups in α- or β-position, close to the ester bond, might hinder the appropriate contact between the lipase molecule and the substrate that must precede the enzymatic hydrolysis or synthesis of the ester. When the methyl branch is further away from the ester linkage, i.e., in the γ position of the fatty acid, the contact with the active center of the lipase seems to be possible as indicated from the good incorporation of 4-methyltetracosanoic acid into glycerides (*cf.* Tables 1 and 2 and Fig. 1).

Balls and Matlack (1938) found that crude pancreas extract rapidly hydrolyzed esters of stearic acid with primary monohydric alcohols, the configuration of the alcohols being without effect (Fig. 2). The splitting of secondary and tertiary ester linkages took place at an almost negligible velocity in comparison with the splitting of a primary ester linkage. The conclusion that only primary ester groups are split by pancreas lipase has been supported in recent investigations by Mattson and Beck (1955). 1-Monoglycerides are hydrolyzed by pancreatic lipase at a higher rate than the corresponding 2-monoglycerides (Schönheyder and Volqvartz 1954). The fact that also 2-monoglycerides are hydrolyzed might be explained by a preceding isomerization to the 1-form.

According to Balls and Matlack one point of attachment for the enzyme on the substrate would appear to be the CO- on

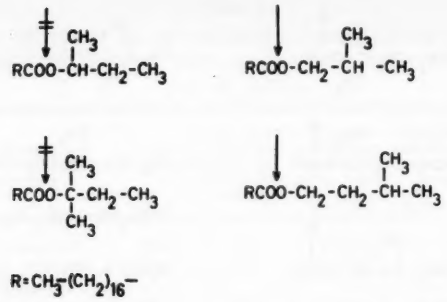

Fig. 2. Scheme of pancreas lipase hydrolysis of stearic acid esters with different alcohols (Balls and Matlack, 1938). ⇟ = hindered enzymic hydrolysis. ↓ = unhindered enzymic hydrolysis.

the alcohol side of the ester linkage. Our results seem to indicate that the $-CH_2-CH_2-CO-$structure on the fatty acid side of the ester linkage (*cf.* Fig. 1) also is of importance for the interaction with the lipase molecule.

Our results are in accord with those obtained by Schulte, Krause and Kirschner (1951) in studies on the velocity of the enzymic hydrolysis of methyl esters of branched, aliphatic acids by a beef-liver esterase. A methyl group in the α- or β-position measurably influenced the reaction velocity while no greater alteration was found when the substituent was far away from the carboxyl. The influence of a methyl branch was less in the β-position than in the α-position (*cf.* Table 2). According to Schulte *et al.* the effect of the α- or β-substituents might be due to a changed polarity of the C : O under the influence of the side chain.

From Table 2 it is seen that the (+)-3D-methylstearic acid was better incorporated into the glycerides by pancreatic lipase than was the 3DL-methylstearic acid. The stereochemical specificity of pancreatic lipase is being further investigated at this institute. Earlier extensive studies in this field (for a review see Ammon and Jaarma 1950) all concern the action of esterases and this seems to be the first demonstration of stereochemical specificity of a real lipase.

However, the fact that different fatty acids are built into glyceride ester bonds with different rates during the hydrolysis with pancreatic enzyme is not only dependent on the enzyme specificty. Thus tributyrin is easily hydrolyzed by

pancreatic lipase (*cf.* Balls and Matlack 1938), but free butyric acid is not incorporated into glycerides by pancreatic lipase to any appreciable extent under the same conditions in the presence of large amounts of water. Neither does free glycerol take part in the formation of glyceride ester bonds under these conditions (Borgström 1954). As pointed out by Bergström and Borgström (1955) a probable explantation of these differences is that butyric acid and glycerol in the free form remain in the water phase and are not in micellar form. Thus they cannot come in contact with the lipase in the micelle surface where triglycerides and free, long-chain fatty acids are closely packed.

A notable finding is that the 8-carbon 5,5-dimethylhexanoic acid is well incorporated into glycerides by pancreas lipase under physiological conditions. Earlier, Borgström (1954 a) has shown that *n*-decanoic acid takes part in this enzymatic formation of ester bonds. It is further noticed (*cf.* Table 1) that *n*-hexacosanoic acid is as well incorporated into glycerides as the ordinary palmitic acid or oleic acid.

Summary.

The substrate specificity of pancreatic lipase has been studied with a series of labelled methyl-branched fatty acids. The incorporation of a fatty acid into glyceride ester bonds by pancreatic lipase is hindered by a methyl group in the α- or β-position of the acid. In contrast one methyl branch in the γ-position of the acid or two methyl branches in the δ-position do not seem to have any influence on the lipase synthesis of ester bonds.

The 8-carbon 5,5-dimethylcaproic acid and the 26-carbon *n*-hexacosanoic acid were both found to be incorporated into glycerides by pancreatic lipase.

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On the Extent of Hydrolysis of Triglyceride Ester Bonds in the Lumen of Human Small Intestine during Digestion.

By

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The percentage of free fatty acids in the lipids recovered from the content of the small intestine during digestion does not necessarily give any direct information on the extent of hydrolysis of the triglycerides fed, as nothing is known about the form in which ingested glycerides are absorbed. The finding that glyceride ester bonds of 2,2-dimethylsubstituted long-chain fatty acids are not hydrolyzed by pancreatic lipase *in vitro* (Bergström, Borgström, Tryding and Westöö 1954), and *in vivo* (Blomstrand, Tryding and Westöö 1956) provides another possibility of calculating the extent of hydrolysis of the ester bonds with normal fatty acids. For this purpose triglycerides containing a small amount of 2,2-dimethyl[1-^{14}C]octadecanoic acid or 2,2-dimethyl[1-^{14}C]nonadecanoic acid have been prepared by biosynthesis or transesterification, respectively. The glycerides were fed in test meals to three normal, adult, male humans. Samples of intestinal contents were collected from different levels of the duodenum and jejunum during the active absorption period. On the assumption that the different glycerides are absorbed at the same rate, we have calculated the extent of hydrolysis of the normal fatty acid ester bonds from the concentration of the labelled 2,2-dimethylbranched acid in the glycerides.

Experimental.

Preparation of the Labelled Material.

Two different, branched-chain fatty acids were used in this investigation. The 2,2-dimethyl[1-^{14}C]stearic acid (0.19 μc/mg, i.e., 160,000 cpm/mg, when assayed in our gas flow counter) was prepared by Bergström, Borgström, Tryding and Westöö, while the 2,2-dimethyl[1-^{14}C]nonadecanoic acid (0.15 μc/mg) was synthesized by Tryding and Westöö (1956). Triglycerides containing 2,2-dimethyl[1-^{14}C]stearic acid were biosynthesized by feeding the free acid dissolved in olive oil to rats with thoracic duct cannulas, from which the lymph was collected. The lymph glycerides were freed from phospholipids by chromatography on silicic acid (Borgström 1952 a) and from free fatty acids on a column of the ion-exchange resin Amberlite IRA-400 (Borgström 1952 b). To ensure that no traces of free acids were present the last procedure was repeated.

The 2,2-dimethylnonadecanoic acid was transesterified with olive oil according to the procedure of Bergström, Borgström and Rottenberg (1952). Thus 2,2-dimethyl[1-^{14}C]nonadecanoic acid (38 mg), olive oil (1 g) and conc. H_2SO_4 (0.01 ml) were mixed and kept at 90° overnight. The reaction mixture was taken up in ether and the unesterified acid was removed by chromatography on Amberlite IRA-400. The free fatty acids were added to another 5 g of olive oil and 0.05 ml of conc. H_2SO_4 and the procedure was repeated. After separation of the free, labelled acids the total glycerides contained 2,700,000 cpm.

Preparation of the Test Meals.

Two different test meals were used.

Test meal A: 1.5 g of the triglycerides containing incorporated 2,2-dimethyl[1-^{14}C]octadecanoic acid were mixed with 13 g of glycerides with incorporated ^{13}C-labelled palmitic acid and 13 g of unlabelled corn oil. This mixture was added to a liquid test meal, containing egg albumin (38 g), glucose (84 g) and water to 620 ml. The fatty acids from this mixture had a specific activity of 85 cpm/5 mg and contained an excess of 0.703 atom per cent of ^{13}C in the carboxyl group.

Test meal B: Olive oil containing transesterified 2,2-dimethyl-[1-^{14}C]nonadecanoic acid (6.0 g) was mixed with corn oil (36.0 g), milk albumin (75 g), glucose (83 g) and water to 800 g. The specific activity of the fatty acids was 113 cpm/mg when 1 mg was plated on each planchet or 57 cpm/mg when 5 mg were plated.

The labelled branched-chain acids comprised only 0.03 and 0.1 per cent of the total fatty acids of the test meals A and B, respectively.

Isotope Determinations.

The determinations of ^{14}C-activity were performed according to usual standard techniques with a gas flow counter ("Tracerlab", background effect: 30 cpm) after direct plating of 1 or 5 mg of the acids on aluminium planchets.

The ^{13}C-content of the fatty acids was determined after decarboxylation of an aliquot by the use of the Schmidt reaction (Blomstrand 1954). The ^{13}C analyses were made with a mass spectrograph at Karolinska Institutet, Stockholm.

Metabolic Experiments.

Sampling Technique.

The subjects included in this investigation were three healthy, male medical students; COE (23 years old), AE (25 years old) and NT (27 years old).

Subjects COE and AE were intubated in the morning with a polyvinyl tubing (inner diameter: 2.1 mm) according to Blankenhorn, Hirsch and Ahrens (1955). When the perforated part of the tubing (over 10 cm in length, about 25 cm from the leading balloon) had reached the duodenum or the upper jejunum, 300 ml of the test meal A were fed.

In subject NT the tubing was allowed to pass through the entire gastrointestinal tract in the hope of obtaining samples from two different levels of the tubing 100 cm apart. When the upper perforated part of the tubing had reached the duodenum, 700 ml of the test meal B were fed. As no free passage was obtained from the anal end of the tubing, samples were only obtained from the upper level, which moved slowly downwards during the collection period.

Samples of the intestinal contents were collected into flasks chilled in ice, which were changed every hour during the 3 to 4 hour collection period. The first sample obtained from subject AE was collected during the first half hour after the ingestion of the test meal.

Extraction and Fractionation.

The volume of each specimen was determined and equal amounts of ethanol, heptane and diethyl ether were added for extraction of the total fat according to Ahrens and Borgström (1956) The upper phases were dried with anhydrous sodium sulfate before evaporation and weighing of the residue. The free fatty acids and neutral fat were separated on a column of Amberlite IRA-400. The neutral fat was saponified and the specific activities of the fatty acids of the different fractions were assayed after direct plating on aluminium planchets.

In two samples (NT-3 and NT-4) the glyceride mixture (100 mg) was separated into tri-, di- and monoglycerides on 10 g columns of silicic acid according to Borgström (1954).

The fatty acids of aliquots of samples from the subjects fed test meal A were decarboxylated by the use of the Schmidt reaction according to Blomstrand (1954). The ^{13}C-excess was determined as described above.

Results and Discussion.

The main results obtained are summarized in Table 1. The specific activities of the glyceride fatty acids recovered from the intestinal contents are 1.4 to 5.0 times as high as the

Table 1.

Summary of the results obtained from the analyses of the samples of intestinal contents.

Sample	Distance from the nose to the site of collection cm	Volume ml	Total fat mg/ml	Ratio between the specific activity of the neutral fat fatty acids of the intestinal contents and the s.a. of the fatty acids of the fed glycerides	Per cent of hydrolysis according to	
					weight of free fatty acids	specific activity of neutral fat fatty acids
NT-1 ...	75—110	25	23.2	2.2	53	55
AE-1 ...	90— 95	88	2.9	2.1	52	52
AE-2 ...	95	200	1.6	2.4	53	58
AE-3 ...	95	126	19.7	1.4	16	29
AE-4 ...	95	130	23.0	1.6	25	38
COE-1 ..	110—125	30	8.8	4.2	71	76
COE-2 ..	125	62	1.0	4.4	70	77
COE-3 ..	125	68	1.9	5.0	75	80
NT-2 ...	120—130	74	7.3	2.5	46	60
NT-3 ...	130—160	60	19.3	2.5	58	60
NT-4 ...	160—180	65	35.6	2.7	66	63

specific activities of the fed glyceride fatty acids. This increase in activity in the glyceride fractions has been attributed to the fact that the ester bonds of the labelled 2,2-dimethyl-branched acids are resistent to the action of pancreatic lipase while the straight-chain fatty acids are partly split off. The specific activities of the free fatty acids in the samples of the intestinal content were found to be insignificant.

On the assumptions that the different glycerides are absorbed at the same rate and that the glycerides fed are not significantly diluted by endogenous glycerides during the digestion, it will be possible to calculate the extent of hydrolysis of the glyceride ester bonds with normal straight-chain fatty acids. That the second requirement is realized in our experiments is shown by the fact that the ^{13}C-excess of the glyceride fatty acids in the intestinal lumen after feeding

test meal A was practically identical with that of the fatty acids of the test meal. The figures for the extent of hydrolysis of the ester bonds of the fed glycerides in the lumen of the small intestine thus obtained are found in Table 1. It is seen that 29 to 80 per cent, with a mean of 59 per cent, of the glyceride ester bonds with normal fatty acids had been split. The percentages of free fatty acids obtained by weight were 16 to 75, with a mean of 53. The agreement of the results obtained in these two different ways indicates that the free fatty acids and glycerides are absorbed from the small intestine at about the same rate.

On the assumptions made above it is possible to calculate also the extent of the complete hydrolysis of the fed triglycerides during digestion, if the composition of the glyceride mixture in the intestinal content is known. This composition has been determined in two samples in the present investigation (NT-3 and NT-4) and found to be in molar percentage: 20, 17 % tri-, 47, 53 % di- and 33, 30 % monoglyceride. If the fatty acid deficit of these glyceride mixtures is calculated, a figure of 38 per cent is found in both cases. A percentage hydrolysis of the fed glycerides over this figure must indicate a complete hydrolysis of triglycerides. If the different glycerides have been absorbed at the same rates, the extent of complete hydrolysis of the fed glycerides in the two samples, with 60 and 63 per cent of the ester bonds split, is calculated to be 36 and 40 per cent, respectively.

The glyceride compositions in our experiments differ somewhat from those obtained in similar experiments by Ahrens and Borgström (1956) in containing more di- and less triglycerides. The samples analyzed in the present investigation were, however, collected considerably lower down in the intestine. The fatty acid deficit of the glyceride mixtures is, however, about the same as that calculated from the figures obtained by Ahrens and Borgström (32 to 40 per cent, mean 37 per cent). The mean value obtained in the present investigation for split glyceride ester bonds, 59 per cent, corresponds approximately to a complete hydrolysis of 35 per cent of the fed triglycerides. These figures are in accord with the results obtained in the rat by several workers (Bernhard, Wagner and Ritzel 1952, Reiser, Bryson, Carr and Kuiken 1952, Morehouse, Skipski, Searcy and Spolter 1956). Our results are also in agreement with the finding by Gidez

and Karnovsky (1956) of free labelled glycerol in the lumen of the small intestine of rat after feeding triolein labelled in the glycerol part.

The results of this investigation show that fed glyceride fatty acids in the human subject are absorbed to a greater extent as free fatty acids than as glycerides. About forty per cent of the triglycerides fed are completely hydrolyzed before absorption. The remaining glycerides are partly hydrolyzed to at fatty acid deficit of around 40 per cent, and the amount of triglycerides absorbed as such is of the order of 10 per cent of those ingested.

Summary.

Glycerides containing a fraction of a percentage of 2,2-dimethyl[1-^{14}C] long-chain fatty acids, the ester bonds of which are resistent to the action of pancreatic lipase, have been fed to human subjects.

From the concentration of the labelled acid in the neutral fat of the intestinal contents it is possible to calculate the real extent of hydrolysis of the glyceride esters fed if it is assumed that the different glycerides are absorbed at the same rate.

The results show that as a mean around sixty per cent of fed glyceride ester bonds are split in the lumen of the small intestine before absorption. About forty per cent of the fed triglycerides are completely hydrolyzed to glycerol and fatty acids, the remaining sixty per cent are partly hydrolyzed to an extent of around forty per cent, giving rise to a mixture of tri-, di- and monoglycerides and free fatty acids.

It is, therefore, clear that fatty acids fed to human subjects in the form of triglycerides are absorbed from the small intestine to a greater extent as free fatty acids than as glycerides.

We are indebted to Prof. S. Bergström for placing the ^{13}C-labelled glycerides at our disposal.

The technical assistance of Mrs. M. B. Nilsvik and Miss K. Sjöström is gratefully acknowledged. The investigation has been supported by grants from Statens Medicinska Forskningsråd, from Knut och Alice Wallenbergs Stiftelse, from the Medical Faculty of the University of Lund and from Statens naturvetenskapliga forskningsråd.

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Lactic Acid Content in Human Venous Blood During Hypoxia at High Altitude.

By

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Lactic acid is formed by anaerobic break-down of carbohydrates — for instance, in cerebral tissue during conditions of oxygen lack (HIMWICK, BERNSTEIN, HERRLICH, CHESLER and FAZEKAS 1942, FAZEKAS and HIMWICK 1943, CRAIG and BEECHER 1943, GUIRDJIAN, STONE and WEBSTER 1944 and 1949, ALBAUM, NOELL and CHINN 1953). Post-mortem determination of lactic acid in human brain tissue has even been applied as a biochemical test to detect hypoxia present prior to aircraft accidents (VAN FOSSAN, WILKS and CLARK 1956).

Hypoxia is one of the factors affecting the level of lactic and pyruvic acids in human blood. When breathing atmospheric air at altitudes of 15,000—18,000 feet, the lactic acid content in the blood slightly increases, the pyruvic acid content remaining unchanged; the levels of both acids increase at altitudes above 18,000 feet (FRIEDEMANN, HAUGEN and KMIECIAK 1945). Apart from this work, we have not been able to find data in the literature on the lactic acid concentration in human blood during hypoxia.

The purpose of this investigation is to determine the degree of hypoxia required to produce increasing concentration of lactic acid in blood; this may indicate corresponding changes in the tissues.

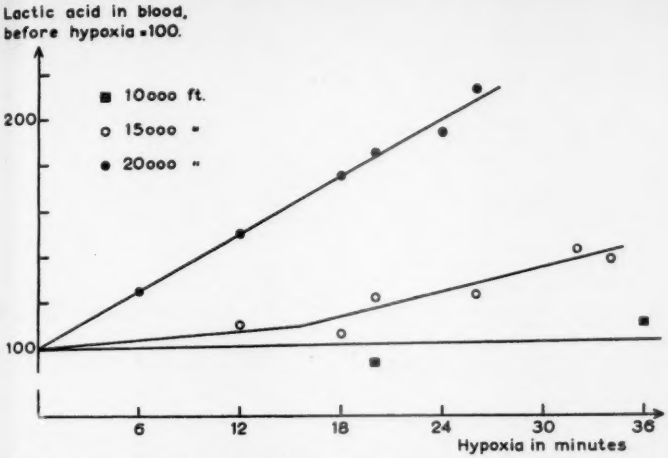

Fig. 1. Lactic acid concentration in human venous blood during hypoxia, breathing atmospheric air at three different altitudes. The points represent the mean values of all experiments at each height, the values being expressed as per cent of the lactic acid concentrations prior to the hypoxic period.

Methods and Experimental Procedure.

Lactic acid is determined by STRÖM's modification (1949) of the method of BARKER and SUMMERSON. Parallel analyses and recovery studies have shown satisfactory accuracy. The results are presented as the mean values of two parallels.

The oxygen saturation of ear blood is determined by oximeter technique, alveolar oxygen and carbon dioxide tensions by analysis of Haldane alveolar samples (Scholander half-cc gas analyzer).

Blood is withdrawn from a cubital vein with a minimum of stasis and completely relaxed forearm. The volume of the sample is adjusted to the 1.0 mark in the syringe, and the contents are expelled in a fine stream through the needle into 20 ml 10 % CCl_3COOH + 0.1 % NaF for subsequent analysis.

The subject is submitted to hypoxia by breathing atmospheric air at 10,000, 15,000, 20,000 or 26,000 feet, respectively, in simulated flights in a low-pressure chamber. He has his usual meal in the morning and rests approximately 20 minutes prior to the experiment, which commences 3—3½ hours after the meal. During ascent, he breathes air + oxygen from an oxygen mask and regulator. During this period, the oximeter has shown normal oxygen saturation of ear blood. Samples of blood are withdrawn once before the mask is removed and at intervals during the hypoxic phase.

Table 1.

Lactic acid concentration in human venous blood during hypoxia, breathing atmospheric air at 15,000 feet.

Subject	Before hypoxia, mgr./100 ml	Hypoxia in minutes						Change during hypoxia	
		12	18	20	26	32	34	mgr./ 100 ml	per cent of value before hypoxia
H. S.	8.0	6.2	6.5		8.3	8.0		0	0
K. B.	7.6	6.1			9.0	8.7		+ 1.1	+ 14
T. A.	9.5		10.2		10.2	10.7	10.7	+ 1.2	+ 13
T. A.	9.5	11.6		11.8				+ 2.3	+ 24
O. K.	7.8	7.8	9.6		9.5	10.2	9.4	+ 2.4	+ 21
M. H.	8.2	9.2	9.3		9.4		11.8	+ 3.6	+ 44
K. B.	7.9				9.1	12.0	11.9	+ 4.0	+ 51
T. A.	8.3				12.9	14.4	13.9	+ 5.6	+ 67
A. H.	6.8	10.5			10.0	13.3		+ 6.5	+ 96
H. S.	9.3	11.3		11.1		15.9		+ 6.6	+ 71

Results and Discussion.

Twenty-two experiments have been carried out on 8 healthy subjects, 20—28 years of age.

When breathing atmospheric air at 10,000 feet, the subject has an alveolar p O_2 of 59 mm Hg (the mean value of 4 experiments), and an oxygen saturation of approximately 88 per cent in ear blood. Alveolar p CO_2 is 39 mm Hg after 2 minutes of hypoxia, and 36 mm Hg after 30 minutes. Fig. 1 shows the mean values of 4 experiments, the values being given as per cent of the lactic acid concentrations prior to the hypoxic period. At this height, there is no change in the level of lactic acid in the blood.

At 15,000 feet, alveolar p O_2 is 44 mm Hg with approximately 78 per cent oxygen saturation of ear blood. Alveolar p CO_2 is 35 mm Hg after 2 minutes of hypoxia, and 33 mm Hg after 28 minutes — the subjects showing only moderate symptoms or signs of hypoxia. As will be seen from Table 1, an unchanging concentration of lactic acid has been found in one experiment — in the others, a questionable or moderate increase. Fig. 1 (the mean values of 10 experiments) indicates a latent period of approximately 15 minutes prior to a gradual increase in the concentration of lactic acid in the blood. FRIEDEMANN et al. (1945) report that 15,000 feet is the lowest height producing increasing concentration of lactic acid in blood — the subject breathing atmospheric air. Our data appear to conform with this view.

Table 2.

Lactic acid concentration in human venous blood during hypoxia, breathing atmospheric air at 20,000 feet.

Subject	Before hypoxia, mgr./100 ml	Hypoxia in minutes						Change during hypoxia	
		6	12	18	20	24	26	mgr./ 100 ml	per cent of value before hypoxia
H. S.	5.1	6.4	7.4	10.4		10.8		+ 5.7	+ 112
H. S.	8.0	10.1	10.1	9.8		13.9		+ 5.9	+ 74
A. H.	4.5	6.0	7.7	8.9			11.0	+ 6.5	+ 144
M. H.	7.1	8.2	9.4		11.3		13.9	+ 6.8	+ 96
O. K.	6.5	6.9	10.2		13.8			+ 7.3	+ 112
M. H.	8.6	14.8	15.6			16.8	17.3	+ 8.7	+ 101
H. S.	8.1	7.7	11.1		15.0		17.1	+ 9.0	+ 111

At 20,000 feet, alveolar p O_2 is 36 mm Hg with approximately 65 per cent oxygen saturation of ear blood. Alveolar p CO_2 is 31 mm Hg after 2 minutes of hypoxia and 27 mm Hg after 22 minutes. The subjects have been severely affected by the hypoxia — with pronounced cyanosis and frequently on the edge of collapse. Table 2 shows an increasing concentration of lactic acid in all experiments. The time required to achieve this increase varies. During the course of the first 6 minutes, no change has been found in 2 experiments, a slight increase in 5, and in 1 experiment — a pronounced increase. Fig. 1 (the mean values of 7 experiments) shows a straight-line increase from the outset of hypoxia.

In one experiment at 26,000 feet, an alveolar oxygen tension of 30 mm Hg was found. The oxygen saturation of ear blood decreased rapidly to below 50 per cent — the subject becoming unconscious within 4 min. 10 sec. 3 samples of blood taken during this hypoxic period showed no change as compared to a sample taken prior to the hypoxia.

The conclusion drawn from these experiments is that the concentration of lactic acid in human venous blood increases during hypoxia, the increase depending on the degree and duration of the hypoxia. Hypoxia of long duration at a height of 15,000 feet leads to an increasing concentration of lactic acid in the blood — the subject being only moderately affected by the hypoxia. At somewhat greater heights (up to approximately 20,000 feet),

the lactic acid concentration increases simultaneously with increasing disability. At even greater heights (more than approximately 25,000 feet), the subject becomes unconscious without changes in the blood because the latent period for increasing lactic acid concentration is longer than that for unconsciousness.

Similar observations have been made by ALBAUM et al. (1953) in determining the lactic acid concentration of brain tissue in rats exposed to anoxia (breathing pure nitrogen through a tracheal cannula). Anoxia gives a profound increase in lactic acid concentration of brain tissue, but unconsciousness and loss of spontaneous electrical activity in the cerebral cortex occurred before significant changes in chemical constituents could be detected.

A biochemical test to detect hypoxia present prior to aircraft accidents based on determination of lactic acid will, therefore, probably be of most value at heights of 15,000 to approximately 20,000 feet, whereas it is of less value at greater heights.

Summary.

The concentration of lactic acid in human venous blood has been determined during hypoxia — the subjects breathing atmospheric air at high altitudes. Lactic acid concentration increases, depending on the degree and duration of hypoxia.

Apart from heights of 15,000 to approximately 20,000 feet, where the lactic acid concentration increases simultaneously with increasing disability, such concentration is poorly correlated to the functional disability during hypoxia.

Acknowledgement.

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The Action of Cortisone on the Mammary Glands of Rats under Various States of Hormonal Imbalance.

By

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It is well established that steroid hormones can stimulate growth and differentiation of the mammary glands of animals with an intact pituitary gland. Information is also available about the degree and type of mammary gland growth promoted by different doses of these agents administered separately or in various combinations during various lengths of time. From these observations it is apparent that the development of the mammary gland depends on a balanced supply of *e. g.* gonadal hormones. Numerous experiments on hypophysectomized animals have further shown that the anterior pituitary gland exerts an important influence on the mammary gland. In the absence of anterior pituitary hormones the responsiveness of the mammary gland to the stimulating actions of the steroidal agents mentioned is severely reduced. The evidence presented so far does not allow a generally valid statement as to whether the result of hypophysectomy is due to removal of anterior pituitary hormones with specific actions on the mammary gland tissues, or to disturbances in the general metabolism. (For lit. see FOLLEY 1952, JACOBSOHN 1954, COWIE and FOLLEY 1955, AHRÉN and JACOBSOHN 1956a).

From what is known about the hormonal control of the intermediary metabolism (*e. g.* ENGEL 1954) and with regard to the metabolic adaptations required generally during growth, it may reasonably be concluded that hormones with powerful metabolic actions play a role in mammary gland growth. In accordance with

this assumption are recent experiments on rats showing that mammary gland growth can be promoted in the absence of anterior pituitary hormones by means of daily injections of long-acting insulin in addition to ovarian steroids (AHRÉN and JACOBSOHN 1956a). With the method used for the assessment of growth a stimulating effect of insulin was hardly apparent in control experiments on rats with an intact pituitary gland.

The actions exerted on the mammary gland by adrenal cortex hormones influencing predominantly the carbohydrate metabolism appear to be more complicated. Thus, SELYE (1954) and JOHNSON and MEITES (1955) observed a stimulating effect of glucocorticoids in rats whilst FLUX (1954) found that cortisone inhibited and ACTH (FLUX and MUNFORD 1957) did not influence mammary gland growth in mice. Preliminary experiments on hypophysectomized rats not included in the present work but reported in a brief review (AHRÉN and JACOBSOHN 1956b) indicated that the action of cortisone on the mammary gland might depend upon a balance which under experimental conditions may, or may not, be established with hormones of other endocrine glands. In addition we observed that cortisone injected into hypophysectomized rats evoked changes in the mammary gland cells independently of whether it was given alone or together with certain other hormones.

The present investigation is an enquiry into the factors determining the type of growth processes resulting from the administration of cortisone. The experiments to be described were performed on hypophysectomized and/or castrated female rats injected with cortisone, ovarian hormones and long-acting insulin in various combinations.

Experimental.

Female rats bred at our Institute were used. Their food, given ad libitum, consisted of bread, dog biscuits, mixed grain and fresh milk supplemented by glucose during the period of injections.

Observations were made on a) hypophysectomized castrated and b) incompletely hypophysectomized and/or castrated rats treated as follows:

Group I: (a: 11 rats, b: 4 rats) Injections of cortisone, 1.0 mg per day (1.0 mg).

Group II: (a: 6 rats, b: 6 rats) Injections of long-acting insulin.

Group III: (a: 3 rats, b: 5 rats) Injections of long-acting insulin and cortisone (1.0 mg).

Group IV: (a: 7 rats, b: 6 rats) Injections of oestrone, progesterone and cortisone (1.0 mg).

Group V: (a: 8 rats) Injections of oestrone, progesterone and long-acting insulin.

Group VI: (a: 13 rats, b: 11 rats) Injections of oestrone, progesterone, long-acting insulin and cortisone in a daily dose of 1) 1.0 mg and 2) 0.1 mg.

Group VII: (a: 5 rats, b: 3 rats) Injections of the suspension medium instead of cortisone but otherwise like group VI or group I.

As far as possible littermates were distributed equally amongst the different groups. The various treatments were, thus, given to a fairly well comparable population of rats living under uniform conditions.

Castration and hypophysectomy were performed under ether anaesthesia, the former at the rat's age of 21 days and the latter about 10 days later. Remnants of hypophysial tissue were searched for by means of microscopic examination of serial sections through the hypophysial capsule and adjacent tissues. The presence of a minute amount of larger, but not typical anterior pituitary cells in contact with pars tuberalis cells was designated as remnant "((+))". Remnants of anterior lobe cells, usually undifferentiated and encapsulated in connective tissue and larger amounts of differentiated pituitary tissues were graded as (+) and +, respectively.

Oestrone and progesterone (Lutocycline, microcryst. 50 mg per ml, Ciba) were injected intramuscularly in doses of 20 μg every other day and 5 mg every 6th day, respectively. Cortisone (Cortisone acetate, 25 mg per ml, Ciba) was given in doses of 1.0 mg or 0.1 mg daily intramuscularly. For the 0.1 mg dose Ciba's preparation was diluted with the suspension medium so that the concentration of cortisone acetate was 2 mg per ml. The volume of the suspension medium given alone to the controls was 0.05 ml. The preparation of long-acting insulin was zinc insulin lente (Novo), 40 i. u. per ml. The dose of insulin injected subcutaneously once daily was successively increased as follows: 1 i. u. during 2 days, 2 i. u. during 4 days, then 4, 6, 8 and 12 i. u., each of these doses during 6 days, subsequently 16 i. u. during about 3 weeks and finally 24 i. u. during one or two weeks. Details about the length of the treatment, determined in a large proportion of the experiments by the capability of the rats to survive, are given below.

Post mortem the following organs were weighed and/or examined: vagina, uterus, adrenal glands, liver, kidneys, heart and lungs. In one or several of the four last mentioned organs inflammatory processes and abscesses were often found after injections with 1.0 mg cortisone during several weeks. In the subcutaneous and intraabdominal fat small whitish patches were also often observed after this treatment. The white spots indicated, as revealed microscopically, a necrosis of fat tissue. Since these destructive processes never involved the mammary glands and since the results obtained from animals suffering from such illnesses did not differ from those of rats remaining healthy, experiments on rats showing abscesses and fat necrosis have not been excluded. It should be emphasized that inflammatory or destructive processes

like those just mentioned never occurred in rats that did not receive a prolonged treatment with 1.0 mg cortisone. This holds true even for littermates kept in the same cage as the rats suffering from illnesses promoted by cortisone.

Before the start of injections, about one month after hypophysectomy, the second right thoracic mammary gland was removed. At different times during the course and/or at the end of the experiment the corresponding left thoracic and later the abdominal glands were taken for whole mount preparation. Microscopic sections were prepared from abdominal glands, if available, and otherwise from the first inguinal gland. A detailed account of the experimental procedures, the histological techniques and the criteria used for the judgement of changes occurring in the mammary glands may be found in previous communications (JACOBSOHN, 1948, AHRÉN and JACOBSOHN 1956a). As in our recent work (1956a) the variations occurring between mammary glands within one group were small. Therefore the prominent features will be described for each group and photographs will show typical examples. As may be seen below, the appearance of whole mount preparations was in several groups dependent upon changes developing inside the ducts. A tabulation of numerical data expected by the reader to indicate the amount of normally occurring mammary gland structures might therefore be misleading. The following tables do not include such data about individual glands.

Results.

The observations made on the mammary glands of the seven groups of rats studied will be described below and typical findings are illustrated by the figures on plates I to IV. Additional information is given by the tables to groups IV—VI.

Group Ia: Injections of cortisone in a daily dose of 1.0 mg were given into 11 completely hypophysectomized rats. The body weight of these rats remained stable or increased moderately, concomitantly with an abnormal deposition of fat both subcutaneously and intraabdominally. In 4 of the 11 rats abscesses were found in the kidneys, liver and/or lungs. Small necrotizing areas were noticed in the fatty tissues of another rat. The weight of both adrenals was reduced to values obtained from corresponding, untreated hypophysectomized rats. The 15 mammary glands examined at different times from 15 to 43 days after the start of injections into the 11 rats of this group presented the same changes as those observed in our previous experiments (1956b) on rats treated with cortisone (1.0 mg). The atrophied mammary glands neither enlarged nor differentiated in a normal manner. From about 3 weeks onwards the whole mounts showed distorted, thick

ducts and side buds, but no end buds (figs. 1 and 2). Thin sections revealed an increase in size and number of the epithelial cells lining the inner walls of ducts, a number of which appeared to be obliterated by the proliferated epithelium. The slightly enlarged, round nuclei, occupying generally an approximately central position in the cytoplasm of these cells, showed a loose network of chromatin and distinct nucleoli. Mitoses were occasionally observed (fig. 13). Secretion was generally slight, but in 3 glands the ducts were fairly widely distended with secretion. In the glands studied earlier than at 18 days the changes just mentioned were not always clearly recognizable.

Ib: Injections were given as in group Ia, but the rats had not been hypophysectomized. The 6 mammary glands obtained from the 4 rats of this group at different times from 11 to 61 days were different from those of group Ia. At 11 days the experimental gland was like the control gland removed before treatment. At 14 days, the only change noticed was the disappearance of end buds present in a considerable number in the control gland of this rat. At 32 days the area covered by the gland was enlarged but differentiation had failed to occur. The ducts, lined by normally appearing epithelial cells, were widely distended with secretion. At 43, 59 and 61 days, respectively, neither growth nor differentiation of the mammary glands were found, and the amount of secretion was less than in the 32 days gland. The thin sections showed, however, that the epithelium lining the inner walls of, foremost, small ducts had increased in size. A proliferation resulting in several epithelial layers or obstruction of ducts was not observed.

Group II: This group comprises 12 rats injected with insulin during periods varying from 6 to 32 days. 6 of these rats were completely hypophysectomized and in 5 others remnants of hypophysial tissue were found at autopsy, 3 of the remnants being small and the other two larger. One of the 12 rats was not hypophysectomized. During the period of injections the body weight of all rats of the present group increased markedly. None of these animals appeared abnormally obese and inflammatory processes were observed neither in the thoracic nor in the abdominal organs. The weight of the adrenal glands was in all of the completely, and in 2 of the incompletely hypophysectomized rats as low as in corresponding, untreated hypophysectomized rats. Except in one incompletely hypophysectomized rat mentioned

below the mammary glands examined after treatment appeared as atrophic as before the injections with insulin. A moderate widening of the ducts due to a slight secretion was observed, however, in some of the rats both without and with remaining hypophysial tissue. The area covered by the mammary gland seemed to be enlarged after treatment of the rats possessing hypophysial remnants. End buds were not observed, however.

From the exceptional rat a peculiar result was obtained. This rat, in which remnants of anterior pituitary cells were found in contact with the pars tuberalis of the hypophysial stalk, was autopsied after 32 days of insulin injections in doses increasing up to 16 i. u. per day. In view of the small size of the hypophysial remnant the weight of both adrenal glands (21.0 mg) appeared high. The whole mount preparation as well as the thin sections showed that the mammary gland was altered in the same manner as in the hypophysectomized rats injected with cortisone (group Ia). In spite of a careful search, an error concerning this experiment, so far exceptional in the whole of our material, could not be revealed.

Group III: Injections of insulin and cortisone (1.0 mg). O the 8 rats included in this group, 3 were completely hypophysectomized, 3 others had minute remnants of pituitary tissue, 1 f rat had a larger remnant and another one was not hypophysectomized. All these rats gained in weight during the period of injections varying from 23 to 65 days, and most of them appeared abnormally obese. Pulmonary abscesses were observed in 4 rats. Small necrotic areas were present in the fatty tissues of 1 rat. The 10 mammary glands examined at different times from 14 to 65 days after the start of injections into the rats without (5 glands) or with minute remnants of hypophysial tissue (5 glands) presented uniform features that developed more and more distinctively with the length of treatment. The ducts and the few side buds grew but little in length and appeared thick. End buds and alveoli were absent (figs. 3 and 4). Microscopic examination of thin sections revealed a more or less abundant proliferation of the epithelium lining the inner walls of ducts, a number of which were slightly distended with secretion (figs. 14 and 15). These changes were essentially the same as those observed in group Ia. One of the hypophysectomized rats, injected during 65 days, should be mentioned specially. The mammary glands of this rat, injected during the last 10 days with 24 i. u. of insulin daily,

Table 1.

Group IV injected with oestrone, progesterone and cortisone (1.0 mg).

Exp.	Age at hyp.ect. days	Body wt. at: first inj. g	Body wt. at: exam. g	First inj. after hyp.ect. days	Period of injections days	Wt. of both adrenals mg
a)						
1	30	70	60 65	34	15 32	5.6
2	36	90	105 100	23	16 26	8.8
3	31	100	85	37	17	7.3
4	29	100	95	39	18	7.8
5	30	110	110 105	34	21 50	8.0
6	30	70	75	37	31	6.9
7	35	75	100 90	23	33 48	6.4
b)						
1[1]	30	90	90 100	34	10 30	5.9
2[1]	29	90	100 105	39	14 30	14.2
3	—	90	140 165	—	14 29	38.4
4	—	105	145 160	—	14 29	44.6
5	—	155	185 215	—	16 31	44.1
6	—	180	205 200	—	24 65	24.2

[1] = incompletely hypophysectomized.

showed a minor number of normally appearing ducts besides those containing heavily proliferated epithelium.

Another rat of the same age, receiving simultaneously the same treatment as the one just mentioned, had a fairly large remnant of pituitary tissue, and this rat showed mammary glands that were different from those described so far for the present group. At 24 and at 65 days ducts widely distended with secretion were found, but a proliferation of duct epithelium was absent (fig. 16). A similar appearance was observed in the mammary gland of the rat with an intact pituitary gland. Here the secretion was less abundant at 23 days, however. The weight of the adrenal glands was considerably decreased in all except the intact rat which showed a slight reduction only.

Table 2.

Group V injected with oestrone, progesterone and insulin.

Exp.	Age at hyp.ect. days	Body wt. at: first inj. g	Body wt. at: exam. g	First inj. after hyp.ect. days	Period of injections days	Wt. of both adrenals mg
1	34	80	95	34	9	10.9
2	33	70	80	33	9	7.9
3	30	85	105	34	10	6.3
4	30	75	100	35	11	7.8
5	29	75	100	39	13	7.0
6	29	65	95	33	14	6.1
7	33	65	95	33	16	8.3
8	29	85	105	39	19	9.8

Group IVa: Hypophysectomized rats injected with oestrone, progesterone and cortisone (1.0 mg): As may be seen from table 1a, the b dy weight of the 7 rats of this group remained roughly unchanged during the period of injections of up to 50 days. The rats of exp. 2, 5 and 7 became very fat. Abscesses were found in the lungs, liver or kidney of all rats except those of exp. 1 and 2. The changes observed in the 11 mammary glands studied at intervals varying from 15—50 days after the first injection were the same as those found in group 1a. The whole mount preparations showed thick ducts and a limited number of short side buds. End buds and acini were absent (figs. 5 and 6). Microscopic examination revealed an increase in size and number of the epithelial cells lining the inner walls of the ducts. In the glands examined after more than 26 days of treatment the epithelium was found to have proliferated into the lumen of the ducts, a number of which appeared obliterated. The nuclei showed a loose network of chromatin and intact nucleoli. In about half the number of glands studied the ducts were slightly distended with secretion (fig. 17). These changes were found in all but one gland (exp. 4) showing, after 18 days of treatment, the same picture as the control removed before the injections. The same was true for another gland (exp. 1) removed at the 15th day. Paraffin sections were, however, not prepared at this instant. The adrenal glands were atrophic.

IVb: Incompletely hypophysectomized rats and rats with intact pituitary gland injected with oestrone, progesterone and cortisone (1.0 mg): Table 1b shows that the body weight of the

Table 8.

Group VI_1, injected with oestrone, progesterone, insulin and cortisone (1.0 mg).

Exp.	Age at hyp.ect. days	Body wt. at: first inj. g	Body wt. at: exam. g	First inj. after hyp.ect. days	Period of injections days	Wt. of both adrenals mg
a)						
1	30	70	80 120	34	10 30	9.3
2	29	70	90 120	33	14 30	9.8
3	36	80	125 140	22	16 30	11.3
4	35	80	90 150	23	22 61	12.1
5	29	95	135	39	30	7.1
b)						
1[1]	29	150	230	39	30	7.6
2[1]	35	80	115	23	31	6.6
3	—	110	180 200	—	14 25	—
4	—	85	140 205	—	14 29	38.4
5	—	90	130 195	—	14 29	40.4
6	—	95	160 205	—	14 29	58.8

[1] = incompletely hypophysectomized.

6 rats increased during the period of injections of 1 or 2 months. As compared with uninjected controls this increase appears markedly limited. The weight of the adrenal glands is in 4 of the 6 rats lower than in untreated rats of our colony. Except in exp. 1, it is, however, considerably higher than in group IVa. The changes observed in the 12 mammary glands examined at different times from 10 to 65 days after the start of injections indicate an extensive proliferation of ducts, end buds and alveoli (figs. 7 and 8). Before fixation milk like secretion could be seen in some of the glands. The whole mounts as well as the thin sections showed ducts and alveoli widely distended with secretion. A proliferation of the epithelium lining the inner walls of these structures was not found in any of the glands of this group (fig. 18). Because of the abundant secretion the degree of mammary gland growth as compared with that occurring in controls injected with oestrone

and progesterone alone cannot be assessed accurately. It may be said, however, that the development of alveoli was increased.

Group V: Hypophysectomized rats injected with oestrone, progesterone and insulin. In all of the 8 experiments of this group the body weight of the rats increased markedly and the mammary glands examined from 9 to 19 days after the beginning of injections presented a clear proliferation of side buds and end buds (table 2, figs. 9 and 10). Slight secretion but no proliferation of duct epithelium was observed. The adrenal glands were atrophic.

Group VI_{1a}: Hypophysectomized rats injected with oestrone, progesterone, insulin and cortisone (1.0 mg). Data concerning this group are given in table 3a. During the period of injections of up to 1 or 2 months the body weight increased considerably and, at autopsy, an excessive amount of subcutaneous and intra-abdominal fat was found. Pulmonary and liver abscesses were present in exp. 2, 4 and 5. The whole mount preparations showed more or less distorted mammary glands with widened ducts and a number of short, thick side buds. End buds were absent (figs. 11 and 12). The cause of this peculiar appearance was revealed by microscopic examination of thin sections showing the same proliferation of the epithelium lining the inner walls of the ducts as in group 1a. In addition to the proliferated epithelium a fair amount of secretion was often present. The abundancy of proliferated epithelium seemed to increase with the duration of the treatment, but normally shaped ducts were reappearing at the same time (figs. 19 and 20). The mammary gland obtained from rat 4 at 61 days contained milk like secretion and a number of small, normally appearing ducts besides a majority of ducts with extensively proliferated epithelium (fig. 21). It should be mentioned that the dose of insulin injected daily into this rat during the last week was 24 i. u. Sections were not studied at 10 days (exp. 1). At 14 days (exp. 2) a proliferation of the duct epithelium was not found.

VI_{1b}: Incompletely hypophysectomized rats and rats with intact pituitary gland treated as the foregoing group VI_{1a}. The experiments are summarized in table 3b. Rats 1 and 2 appeared very fat, no. 6 had small whitish patches indicating necrotic areas in the subcutaneous and intraabdominal fat. Otherwise, inflammatory processes were not found in the rats of this group. The changes occurring in the mammary glands were strikingly different from those seen in the foregoing group VI_{1a}. Already after 2 weeks

Table 4.

Group VI_2 injected with oestrone, progesterone, insulin and cortisone (0.1 mg).

Exp.	Age at hyp.ect. days	Body wt. at: first inj. g	Body wt. at: exam. g	First inj. after hyp.ect. days	Period of injections days	Wt. of both adrenals mg	Remnant of ant. pit. gl.
1	33	60	75	33	6	—	⊖
2	32	65	75	29	8	—	⊖
3	34	85	105	34	11	8.6	⊖
4	34	75	110	34	11	8.9	⊖
5	32	80	85	26	11	10.0	⊖
6	33	65	95	33	14	8.2	⊖
7	33	70	100	33	17	8.6	⊖
8	31	80	120	32	17	8.5	⊖
9	32	85	95	29	9	9.0	((+))
10	33	75	110	33	14	8.9	((+))
11[1]	—	170	180	—	9	—	
12[1]	—	135	175	—	11	34.9	
13[1]	—	135	205	—	17	32.8	

[1] = not hypophysectomized.

of treatment a considerable growth of ducts, side buds, end buds and alveoli was obtained. Most of the glands contained a fair amount of secretion. Abundant, milk like secretion was present in exp. 1. In exp. 2, as well, the secretion appeared like milk. A proliferation of the epithelium lining the inner walls of ducts and acini was not found. In all but exp. 6 the weight of both adrenal glands was lower than normal.

Group VI_2: Injections of oestrone, progesterone, insulin and cortisone (0.1 mg): Table 4 shows that this group includes 8 completely hypophysectomized rats, 2 rats with extremely small remnants of hypophysial tissue and 3 others with an intact pituitary gland. During the period of injections varying from 6 to 17 days the body weight increased about as much as in the corresponding rats of group V. The growth processes observed in the mammary glands of the completely hypophysectomized rats (exp. 1—8) and of that of exp. 10 with a minute hypophysial remnant were roughly the same as in group V (figs. 22 and 23). In exp. 9 and in the 3 experiments (11—13) on rats that were not hypophysectomized the mammary glands appeared larger and more differentiated than in the other rats of this group. Microscopic examination of thin sections performed in all but exp. 1 revealed a slight

amount of secretion but no abnormalities of the epithelium lining the inner walls of the ducts (fig. 24). The weight of both adrenal glands of rats 3 to 10 was as much reduced as in group V and in exp. 12 and 13, it was lower than normal. Neither inflammatory processes nor abscesses were observed in the intrathoracic or abdominal organs of the rats of this group.

Group VII: This group includes experiments on rats treated as group VI or group I, except that instead of the cortisone (1.0 mg) its suspending medium was injected. Thus, 3 hypophysectomized rats and 2 rats with intact pituitary gland received injections of oestrone, progesterone, insulin and the suspension lacking cortisone (controls to group VI). Another 2 hypophysectomized rats and 1 rat with an intact pituitary gland were injected with the suspension alone (controls to group I). The results obtained from these control experiments will be given briefly. Both groups reacted as if they had not been injected with the control solution at all. The mammary glands examined from the controls to group VI presented the same response as those of group V and of glands of similarly treated rats with intact pituitary gland, respectively. No changes occurred in the mammary glands of the rats injected with the suspending medium alone. The epithelium lining the inner walls of ducts had neither increased in size nor proliferated in any of these mammary glands, 5 of which were examined after 31 days of injections.

Summary of Results and Discussion.

In view of the question raised in the introduction the present experiments will be discussed in the following order: A: Experiments on hypophysectomized castrated rats 1) injected with cortisone (group I) or insulin (group II) or cortisone + insulin (group III) and 2) treated as before but given in addition, oestrone and progesterone (group IV, V, VI). B: Experiments as in A_1 and A_2 but performed on castrated rats possessing functionally active anterior hypophysial tissue.

A_1: The results obtained from group Ia (figs. 1, 2, 13) show that cortisone (1.0 mg per day) does not promote normal mammary gland growth when given alone to hypophysectomized rats. However, the enlargement and proliferation of epithelial cells lining the inner walls of ducts, and the secretion present in the ducts indicate that cortisone did stimulate the growth and activity

of essential components of the mammary gland. This stimulating action observed regularly after prolonged treatment was exerted by cortisone in the absence of ovarian and anterior pituitary hormones. As to the mammary gland, the conditions were such that the main growth promoting hormones, ovarian steroids, prolactin and growth hormone were lacking (LYONS, JOHNSON, COLE and LI 1955). As to the intermediary metabolism, the main factors, growth hormone and insulin, counterbalancing by their net effect on body protein the negative effect of cortisone, were absent and insufficient, respectively (*e. g.* ENGEL 1954, RUSSEL 1955). To our knowledge the effect of cortisone on the mammary glands of hypophysectomized rats has not been described previously by others (cf. AHRÉN and JACOBSOHN 1956b).

The observations made on the hypophysectomized rats of group II confirm those of AHRÉN and JACOBSOHN (1956a) studying rats that at hypophysectomy were older than the present ones. Further, the present group II showed that insulin treatment alone may result in slight secretion, but neither in obvious growth nor in any abnormal proliferation of the duct epithelium. As to the question about the growth of the mammary glands it should be pointed out that our methods do not allow conclusions concerning minor differences. For discussion of the action of insulin in the hypophysectomized rat see SALTER and BEST (1953) and AHRÉN and JACOBSOHN (1956a).

With regard to our previous findings (cf. group V) group III was studied in order to see whether a treatment of the hypophysectomized rats with insulin would enhance or alter the effect of cortisone. The changes occurring in the mammary glands of the hypophysectomized rats of group III appeared, however, to be essentially the same as in group Ia. This holds true for the period of injections of up to 43 days studied in group Ia, and attention must be paid to the fact that the extent of the epithelial proliferation could not be accurately assessed. With this in mind, it may safely be said that, if anything, the proliferative changes were more extensive in group III injected with insulin in addition to cortisone. The prolonged treatment given in group III (up to 65 days) resulted in a clearly increasing proliferation of the duct epithelium (figs. 14 and 15). In addition, at least in one rat, small ducts of normal appearance were found. As mentioned under "results" the dose of insulin administered by that time was 24 i. u. Since the dose of cortisone (1.0 mg) was kept the same throughout

the experiments whilst that of insulin was gradually increased, the balance between the two hormonal agents should eventually turn in favour of insulin. This seems to have happened in that rat.

A_2: The effect of the combined actions of cortisone (1.0 mg), oestrone and progesterone on the mammary glands of hypophysectomized rats (group IVa) will be compared 1) with that obtained from group Ia injected with cortisone alone and 2) with observations made on group V injected with insulin instead of cortisone.

Considering the shortcomings of our methods in evaluating quantitative differences it seems clear that the mammary glands of group IVa (figs. 5, 6, 17) could not be distinguished from those of group Ia (figs. 1, 2, 13). Thus, the addition of oestrone and progesterone altered neither the response of the mammary gland to cortisone, nor did cortisone change the reaction of the mammary gland to oestrone and progesterone. As pointed out previously (AHRÉN and JACOBSOHN 1956b) and confirmed in the present work the action of cortisone on the responsiveness of the mammary gland to ovarian steroids appears essentially different from that of insulin. In group V (figs. 9, 10) injected with oestrone, progesterone and insulin a distinct proliferation of normal appearing structures was found in all glands. This result confirms those obtained previously (AHRÉN and JACOBSOHN 1956a) from rats hypophysectomized at a more advanced age than the present rats. The mammary glands of the present rats were considerably less developed at the start of the injections of insulin, the dose of which was less rapidly increased than in the rats studied earlier. Therefore, after treatment the mammary glands appeared smaller and less differentiated than those described and illustrated in our previous work. Growth and differentiation appeared, however, to be considerably more extensive in the glands of group V than in those of group VI_{1a} treated with cortisone (1.0 mg) in addition to oestrone, progesterone and insulin. Compared with the observations made on the foregoing groups the glands of group VI_{1a} (figs. 11, 12, 19, 20, 21) seem to show a variety of changes resulting from competitive actions, on one hand, of oestrone, progesterone + insulin and, on the other, of cortisone, neither part being able to suppress the other one. All glands of group VI_{1a} showed ducts with enlarged and proliferated epithelium, general mammary gland growth occurred but appeared to be reduced when compared with group V, and secretion was more abundant than in any of

the groups hitherto commented upon. Thus, cortisone seemed to be dominating and effective in 3 different ways 1) independently of, 2) antagonistically to and 3) synergistically with the combined actions of oestrone, progesterone + insulin. The balance between cortisone and the other hormonal group seemed in some of the rats (VI_{1a}) to be shifting at about one month of treatment, that is, when the dose of insulin had been increased to 16 i. u. or more. The mammary gland examined at 61 days was distended with milk like secretion and showed a considerable number of small, apparently newly outgrown, normal ducts besides those with proliferated epithelium.

Since it seemed neither desirable nor feasible to reach an equilibrium by further increasing the dose of insulin, the dose of cortisone was reduced to one tenth instead. The result obtained from the hypophysectomized rats of group VI_2 (figs. 22, 23, 24) injected with 0.1 mg cortisone showed a dominance of the combined effect of oestrone, progesterone + insulin. In fact, the glands appeared so similar to those of group V (figs. 9, 10) that, at first sight, it appears doubtful whether the 0.1 mg dose of cortisone exerted any effect at all. However, that this dose is not without effect is indicated by unpublished findings of proliferated epithelium in mammary glands of young rats injected with cortisone (0.1 mg) and insulin up to 4 i. u., as well as by the reduction of the adrenal weight observed in the intact rats of group VI_2.

Further evidence supporting the assumption that the effect of cortisone on the mammary gland can be counteracted and/or altered by hormones of other endocrine glands was sought for and obtained from the experiments to be discussed under "B" on castrated rats with remaining anterior hypophysial tissue.

B: In spite of the fact that the experiments on the rats with intact hypophysial tissue were performed concomitantly with and using the same substances and dosages as in those commented on above the action of cortisone on the mammary gland revealed itself in a different manner. The stimulating effect on the growth of the duct epithelium was absent in all but group Ib. In this group enlarged, but not proliferated cells were found lining the inner walls of ducts of glands examined after prolonged treatment with 1.0 mg cortisone alone, that is after 43 to 61 days. The inhibitory effect on the growth of the mammary gland could not be evaluated in the rats of group Ib and group III with remaining hypophysial tissue. A stimulation of the secretory

activity was noticeable in group Ib and clearly apparent in the rats mentioned of group III (fig. 16) injected with cortisone + insulin. Stimulation of mammary gland secretion was also observed in group IVb (fig. 18) and VI_{1b} receiving oestrone and progesterone, and oestrone, progesterone + insulin, respectively, in addition to cortisone. In both these groups two of the glands contained milk like secretion. Furthermore, group IVb (figs. 7, 8) and VI_{1b} indicated that the growth inhibiting effect of cortisone was not only abolished, but, at least partly, inversed. The development of acini was more abundant than in the mammary glands of rats injected with the same hormones except cortisone. The observations made on group IVb agree with those of SELYE (1954) and JOHNSON and MEITES (1955) studying rats with an intact pituitary gland. It should be emphasized, however, that cortisone did not induce mammary gland growth when given alone (Ib) or together with insulin (III), and that growth processes other than the development of acini could not be ascertained with sufficient accuracy in group IVb and IV_{1b}.

From the experience gained in the present investigation it seems clear that the effect elicited by cortisone in the mammary gland should be analysed with due regard to the endocrine state of the animal both as to its effect on the structures of the mammary gland and to the consequences resulting from an eventual upset of the general metabolic equilibrium. On such a basis the discrepant observations of SELYE (1954), JOHNSON and MEITES (1955) and FLUX (1954) might find an explanation. The two groups of authors, mentioned firstly, studied mature rats with an intact pituitary gland. SELYE's rats were castrated and adrenalectomized, but treated with oestradiol and cortisol. The rats of JOHNSON and MEITES had intact ovaries. In the experiments of these workers the mammary glands received, presumably, considerably stronger growth stimuli than the glands of FLUX's young castrated mice injected with a small dose (0.02 μg) of oestrone, but, calculated from body weight, approximately the same amount of cortisone. From the illustrations of whole mounts given by FLUX, 1954, it seems as if the effect of cortisone obtained on the young mice was similar to that observed in the present experiments (group VI_{1a}) on hypophysectomized rats injected with oestrone, progesterone, insulin and cortisone (1.0 mg). Thus, in the experiments of FLUX cortisone appears to have been dominating and in those of the other authors mentioned the balance

seems to have favoured hormones with opposed actions. That mice are more sensitive than rats to metabolic actions of cortisone is indicated by the effects of cortisone in these two species on the deposition of glycogen in the liver (LONG, KATZIN and FRY 1940).

With regard to the possible role played by glucocorticoids in mammary gland growth and lactation under physiological circumstances the following observations appear relevant: 1) The growth of normal mammary gland structures was inhibited or impeded when the metabolic actions of cortisone could not be counteracted efficiently. The mechanisms underlying this effect of cortisone may reasonably be assumed to be similar to those causing the well known inhibition or retardation of general body growth (*e. g.* ENGEL 1954). 2) Under the conditions mentioned under "1)" and/or when the mammary growth promoting actions of other hormones had been reduced or eliminated, cortisone evoked an abnormal growth of mammary gland cells normally predestined to functional activity. That an abnormal increase in size and number of epithelial cells lining the inner walls of ducts can, under such conditions, result from actions of hormones manufactured in the adrenal glands was observed by JACOBSOHN (1949) in experiments on parabiotic rats, and is probably the explanation of the exceptional result obtained from the incompletely hypophysectomized rat of the present group II. So far, no explanation can be given for the mechanism leading to the peculiar alteration of these epithelial cells. 3) Under conditions optimal as to mammary gland development and maintenance of homeostasis the predominant actions of cortisone were enhancement of alveolar growth and stimulation of secretion. Hence, it seems reasonable to assume that these two effects indicate the physiological role of glucocorticoids. Further support for this assumption may be found in work of LYONS, LI and JOHNSON (1952) and LYONS, JOHNSON, COLE and LI (1955).

The effects mentioned under "results" as to body growth, deposition of fat, adrenal weight, occurrence of diseases indicated by the presence of abscesses and inflammatory processes in some of the intrathoracic and abdominal organs and the findings of necrotic areas in fatty tissues indicate that the dose of 1.0 mg cortisone per day should be regarded as high, at least when administered during periods of several weeks. A discussion of these, except for fat necrosis, well known effects reviewed by *e. g.* NOBLE (1955) seems unnecessary. An effect similar to that observed

in the fatty tissues of some of the present rats has, to our knowledge, not been described previously, and further work is required to substantiate this finding.

Summary.

The action exerted by cortisone on the mammary glands of castrated female rats was studied under various endocrine conditions. The main results were as follows:

A) Pituitary gland removed:

1) Cortisone promoted enlargement and proliferation of the epithelial cells lining the inner walls of the ducts. Normal growth and differentiation did not occur.

2) The effect of cortisone + oestrone and progesterone was roughly the same as in "1)".

3) The growth of normal structures occurring after treatment with oestrone, progesterone + long-acting insulin was markedly reduced by cortisone. The effect on the duct epithelium described under "1)" predominated.

B) Pituitary gland intact:

1) Cortisone stimulated secretion, but not growth. After prolonged treatment the duct epithelium appeared enlarged, but did not proliferate.

2) Cortisone + oestrone and progesterone promoted growth and abundant secretion. The development of acini appeared enhanced by the addition of cortisone to the ovarian steroids. Abnormalities of duct epithelium were not found.

3) The result obtained after treatment with cortisone, oestrone, progesterone + long-acting insulin was similar to that of "2)", but secretion appeared more abundant.

The observations made on a total of seven groups of rats subjected to different treatments are summarized and discussed with regard to the dependence of the mammary gland response to cortisone upon the endocrine balance both as to effects on mammary gland structures and the general metabolism.

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Comments to Plates I—IV and legends on pages 273 and 274.

Comments to Plates I—IV.

The figures illustrate observations made on mammary glands of the present groups of rats. The glands shown in figs. 7, 8, 16 and 18 belonged to rats with intact hypophysial tissue. All the others were obtained from hypophysectomized rats. Figs. 1—12, 22 and 23 are photographs of whole mount preparations. Second thoracic glands are shown in all these figures but nos. 4, 8 and 12 giving abdominal glands. Figures 13—21 and 24 are microphotographs of 10 μ thick sections through abdominal or first inguinal glands stained with hematoxylin eosin. All whole mount preparations and all sections were taken with the same magnification, respectively (see plate IV). The experiment and prominent features of the mammary gland illustrated are briefly indicated below.

Fig. 1: Group Ia. Atrophic gland removed 29 days after hypophysectomy (control to fig. 2, 29 days after h.e.).

Fig. 2: Group Ia. Corresponding gland of the same rat as in fig. 1 (same rat as fig. 1) after daily injections of 1.0 mg cortisone during 19 days (*cortisone*, 19 days). Ducts slightly thickened. No growth of side buds or end buds.

Fig. 3: Group IIIa. Control to fig. 4, 19 days after h.e.

Fig. 4: Group IIIa. Same rat as fig. 3. *Insulin* and *cortisone*, 46 days. Abdominal gland covering a larger area than the thoracic control. Thickened ducts, no differentiation. End buds absent.

Fig. 5: Group IVa, table 1a, exp. 6. Control to fig. 6, 30 days after h.e.

Fig. 6: Group IVa. Same rat as fig. 5. *Oestrone, progesterone* and *cortisone*, 31 days. Thickened ducts. No growth of side buds or end buds.

Fig. 7: Group IVb, table 1b, exp. 3. Control to fig. 8, 6 days before the start of injections. Not hypophysectomized.

Fig. 8: Group IVb. Same rat as fig. 7. *Oestrone, progesterone* and *cortisone*, 29 days. Part of abdominal gland showing marked growth and differentiation.

Fig. 9: Group V, table 2, exp. 6. Control to fig. 10, 29 days after h.e.

Fig. 10: Group V. Same rat as fig. 9. *Oestrone, progesterone* and *insulin*, 14 days. Growth and differentiation. Side buds and end buds present.

Fig. 11: Group VI_{1a}, table 3a, exp. 3. Control to fig. 12, 19 days after h.e.

Fig. 12: Group VI_{1a}. Same rat as fig. 11. *Oestrone, progesterone, insulin* and *cortisone*, 30 days. Abdominal gland covering a larger area than the thoracic control. Thickened ducts and a number of side buds. No end buds. (The thoracic gland examined after 16 days was not enlarged and showed a very limited number of side buds only. The ducts appeared thickened.)

Fig. 13: Group 1a, cf. fig. 1. Ducts with enlarged and proliferated epithelium after 32 days of injections with *cortisone* (1.0 mg). Slight secretion.

Fig. 14: Group IIIa, cf. fig. 4. Abundant proliferation of duct epithelium after 65 days of injections with *insulin* and *cortisone*. The diameter of the ducts is enlarged and their lumina appear obstructed. Note the peculiar arrangement of the enlarged epithelial cells.

Fig. 15: Group IIIa, same rat as in fig. 3, but examined after 62 days of injections of *insulin* and *cortisone*. Transversal section showing proliferated epithelium and slight secretion. A few ducts with smaller epithelium are apparent within the lower cluster of ducts.

Fig. 16: Group IIIb. Rat with large remnant of pituitary gland. Treatment during 65 days as in figs. 14 and 15. Area including a large duct. Note secreting epithelium and abundancy of secretion distending the ducts. Enlargement and proliferation of duct epithelium were not found.

Fig. 17: Group IVa, same rat as in fig. 5. Injections of *oestrone, progesterone* and *cortisone* during 31 days. Marked proliferation of duct epithelium. Some secretion within the ducts.

Fig. 18: Group IVb, same rat as in fig. 7. Injections as in fig. 17 during 29 days. Note the ducts and acini, lined by normal epithelium and widely distended with

secretion. The reaction of this rat with intact pituitary gland is qualitatively and quantitatively different from that of the hypophysectomized one of fig. 17.

Fig. 19: Group VI_{1a} (Table 3a, exp. 2) *Oestrone, progesterone, insulin* and *cortisone* during 30 days. Large cluster of ducts slightly distended with secretion and with markedly proliferated epithelium showing a disorderly arrangement. The small part of another cluster to the lower right shows ducts lined by one layer of only slightly enlarged epithelial cells.

Fig. 20: Group VI_{1a}, same rat as in fig. 11. Injections as in fig. 19 during 30 days. Longitudinally cut duct with enlarged and proliferated epithelium and some secretion. The outgrowths at the lower end of this duct are lined by small, normal appearing cells.

Fig. 21: Group VI_{1a}, table 3a, exp. 4. Injections as in figs. 19 and 20, but during 61 days. Transversal section through two clusters of small ducts lined by one row of small, normal appearing epithelial cells. A majority of ducts, not shown here, presented highly abnormal epithelium.

Fig. 22: Group VI_2, table 4, exp. 4. Control to fig. 23, 21 days efter h.e.

Fig. 23: Group VI_2. Same rat as fig. 22. *Oestrone, progesterone, insulin* and *cortisone* (0.1 mg), 11 days. The growth and differentiation of this gland show that one tenth of the dose of cortisone administered in the other groups does not inhibit mammary gland growth (cf. fig. 10, group V).

Fig. 24: Group VI_2, table 4, exp. 7. Same treatment as in fig. 23, but during 17 days. Small ducts distended with secretion and lined by one layer of normal appearing epithelial cells.

Plate I.

Fig. 1.

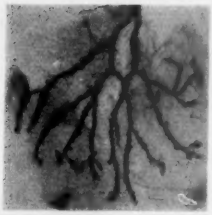


Fig. 2.

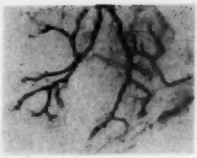
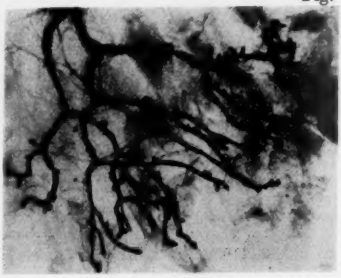


Fig. 3.



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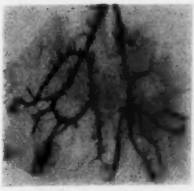

Fig. 5.

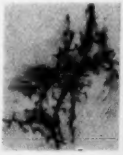

Fig. 7.

Fig. 8.

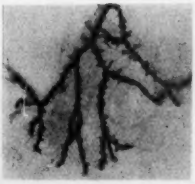

Fig. 6.

Fig. 9.

Fig. 10.

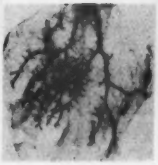

Fig. 11.

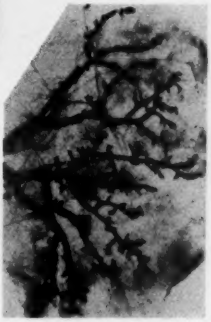

Fig. 12.

K. Ahrén and Dora Jacobsohn: Action of Cortisone on the Mammary Glands.

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Plate II.

Fig. 13.

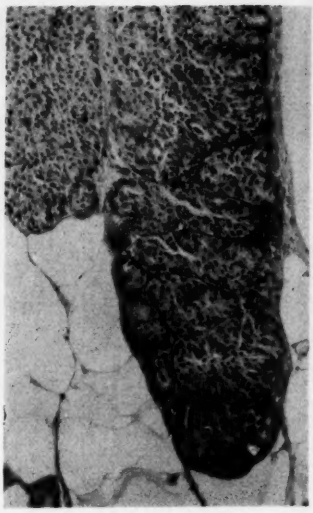

Fig. 14.

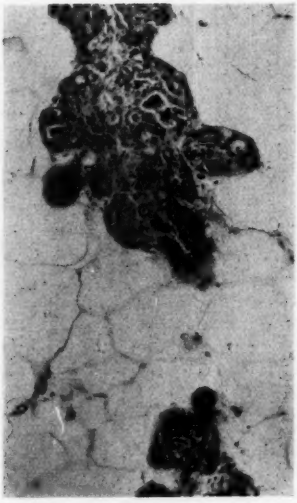

Fig. 15.

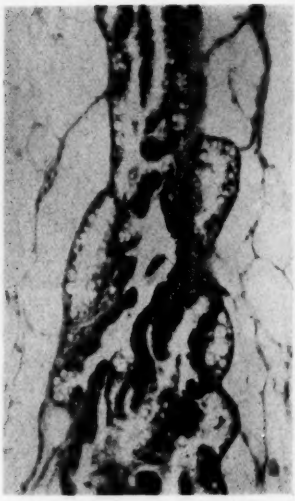

Fig. 16.

Plate III.

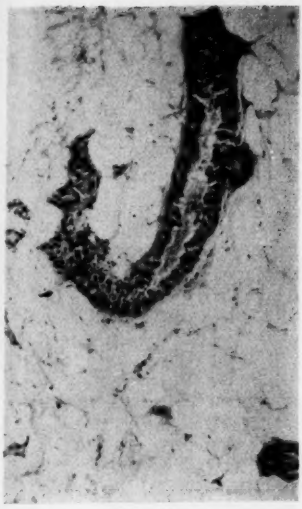

Fig. 17.

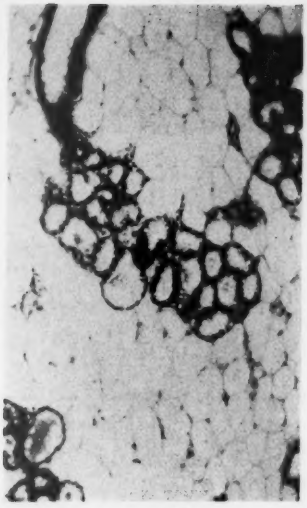

Fig. 18.

Fig. 19.

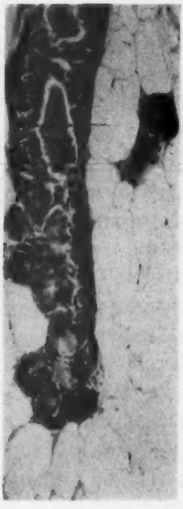

Fig. 20.

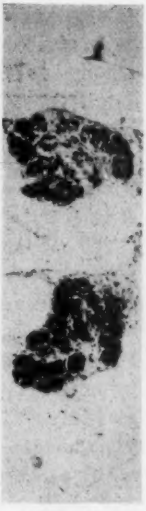

Fig. 21.

K. Ahrén and Dora Jacobsohn: Action of Cortisone on the Mammary Glands.

Acta physiol. scand. 40: 2—3.

Plate IV.

Fig. 22.

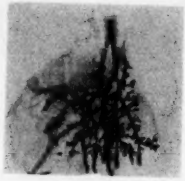

Fig. 23.

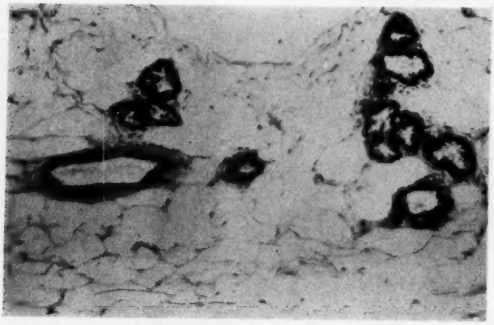

Fig. 24.

5 mm (whole mounts)

0.5 mm (microphotographs)

From the Department of Pharmacology, University of Oslo, Norway.

The Action of Tetraethylammonium (TEA) on the Rat Diaphragm.

By

JACOB STOVNER.

Received 8 April 1957.

The autonomic ganglionic blockade produced by tetraethylammonium (TEA) has been fully elucidated by ACHESON and PEREIRA (1946 b). However, these authors state that TEA "over a wide range of doses has no other than a specific ganglionic blocking action". This statement is true as far as neuromuscular blocking action is concerned, as ING and WRIGHT (1933) and ATKINSON (1952) have emphasized that this action is very slight. ING (1936) compared the action of a number of simple quaternary ammonium ions on frog nerve-muscle preparations with respect to "curariform" activity and found this to be minimal for TEA. Fibrillary twitchings and irregular muscle contractions in frog nerve-muscle preparations immersed in concentrations of TEA higher than 10 mM had been noticed by several earlier workers (MARSHALL 1916, LOEB and EWALD 1916). MARSHALL regarded the observed muscle twitchings as an action at the myoneural junction. Increased excitability after treatment of frog nerves with TEA in concentrations above 10 mM was convincingly demonstrated by COWAN and WALTER (1937), and this could be antagonized with calcium. An action on frog nerve activity has also been observed by LORENTE DE NÓ (1948, 1949). He found that TEA and several related compounds restored conduction in certain types of frog nerve fibres after conduction failure had been produced by sodium depletion.

HAGIWARA and WATANABE (1955) have shown that concentrations of TEA above 12 mM applied to the muscle membrane of the toad caused prolongation of the muscle action potentials with repetitive spikes in response to single nerve stimuli.

The effect of TEA on the mammalian neuromuscular system has been studied by ACHESON and PEREIRA (1946 a), ATKINSON (1952) and JEPSON, SIMEONE and LYNN (1953). These workers found no significant effect of TEA in doses below 10 mg per kg injected intraarterially in cats and dogs. With higher doses a transient neuromuscular depression occurred preceded by a small increase of the muscle responses to both direct and indirect stimulation.

Although the normal mammalian neuromuscular junction is thus shown to be unaffected by lower doses of TEA, a profound effect becomes apparent under abnormal conditions. When the transmission is depressed by some specific neuromuscular blocking agents or by a deficiency of calcium in the suspending medium, TEA has a marked restoring effect, as reported briefly by KENSLER (1949, 1950) and SULLIVAN and KENSLER (1950). No further report on the restoring effect of TEA on the various forms of neuromuscular block has since appeared in the literature. Further investigation into this was therefore planned by the author using both electro-physiological methods and testing the chemical sensitivity of the postsynaptic membrane.

Before this could be done, however, it proved necessary to carry out an investigation of the action of TEA on an unaffected mammalian nerve-muscle preparation, because this had not been reported on before. The isolated phrenic nerve diaphragm preparation from the rat was chosen, because this has proved adaptable both for electrophysiological technique (HADJU and KNOX 1950) and for testing chemical sensitivity (BURGEN, DICKENS and ZATMAN 1949).

The investigation was planned to study certain actions of TEA on the unaffected neuromuscular transmission of this mammalian nerve-muscle preparation. It was further planned to investigate the effects of TEA on the action potentials of the phrenic nerve and diaphragm muscle, as well as the electrical and chemical excitability of the diaphragm muscle. The results thus obtained would form the basis for further study of the influence of TEA on the various forms of neuromuscular block.

Methods.

a) Contractions of Phrenic Nerve Diaphragm Preparations.

The isolated phrenic nerve diaphragm preparation from adult rats was used (BÜLBRING 1946). The bath fluid (100 ml) was a Tyrode solution of the following composition given in g per 1000 ml: 2 g dextrose, 1 g Na HCO_3 8 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.05 g Na H_2PO_4 and 0.01 g $MgCl_2$.

The temperature of the bath was kept constant at either 37° C or 22° C. The bath was aerated with 95 % O_2 and 5 % CO_2. The pH of the bath fluid was kept constant between 7.3 and 7.4.

A section of the right or left diaphragm with a piece of the phrenic nerve was suspended in the bath. It was found convenient to place the tendinous end downwards fixed to the bottom of the bath, as first described by BURGEN et al. (1949) for close intravascular injection, and also used by BERGH (1953). A thread was tied to the costal margin and attached to a lever. Isotonic contractions were recorded by a lever with a 6 : 1 advantage. When testing the excitability of the muscle to direct stimulation, a thin cellophane point writing lightly on the drum with minimal friction was attached to the point of the lever.

Platinum electrodes for stimulation of the nerve (BÜLBRING 1946) were, in most experiments, immersed in the same bath as the preparation. Some experiments were performed in which the nerve was stimulated in a small glass chamber in which the nerve passed through a hole in the bottom sealed off with wax. The fluid surrounding the nerve at the point of stimulation was thereby separated from the fluid in contact with the rest of the preparation, thus making it possible to study the effect of a drug on the nerve alone.

A stimulator delivering rectangular impulses of various durations was employed. For indirect stimulation frequencies from 6 per min to 100 per sec and a pulse length of 0.3 msec were used. For direct stimulation of the muscle one platinum electrode was placed along the costal margin as the anode, and another at the tendinous end as the cathode, both immersed in the Tyrode solution. The preparation was fully curarized. The frequency of stimulation was 6 per min. The first noticeable movement of the lever with progressively stronger stimuli was regarded as the threshold value. Control readings were taken for various pulse lengths.

b) Close Intravascular Injection of Acetylcholine and Potassium in the Isolated Rat Diaphragm Preparation.

The method employed by BURGEN et al. (1949) was used with only slight modifications. Instead of a cannula, a polythene tube was tied into the thoracic vena cava and fixed to a bar at the bottom of the bath. The acetylcholine and KCl for injection were dissolved in normal Tyrode solution adjusted to pH 7.4 and 37° C before injection. The

volume of fluid injected was usually 0.2 ml. A rapid muscle twitch of the same height as the response to a supramaximal nerve stimulus was obtained in many preparations by injection of doses as small as 2 micrograms acetylcholine or 0.5 mg KCl. The injection of 0.2 ml normal Tyrode only produced a slight muscle twitch due to the physical expansion of the preparation. The degree of leakage from the cut sides of the preparation would influence the height of the muscle twitch as would of course the speed of injection. However, by injecting as fast as possible each time with a 1 ml syringe containing only 0.2 ml volume of fluid, muscle twitches of fairly constant height could be produced. Gross changes in the stimulant action of acetylcholine or KCl could easily be detected with this method.

c) Action Potentials from the Phrenic Nerve and Diaphragm Muscle of the Rat.

The full length of the phrenic nerve from the neck to the diaphragmatic insertion was dissected under a magnifying glass. The nerve was mounted on the stimulating and recording electrodes and immersed in Tyrode solution. During the recording the nerve was lifted out of the bath. The nerve was crushed between the recording electrodes in order to obtain a monophasic action potential.

The rat diaphragm with part of the phrenic nerve was fixed horizontally over a triangular opening in a cork mat (cf. HADJU and KNOX 1950). The platinum electrode was fused into a perspex tube attached to a screw adjustment allowing horizontal movement over the muscle. The reference electrode was in the bath. Both stimulating and recording electrodes were immersed in Tyrode solution during the recording.

The action potentials from both nerve and muscle were recorded on a cathode ray oscilloscope after applying single supramaximal stimuli to the nerve. The bath fluid was Tyrode solution of the above composition kept at 37° C and aerated with carbogen.

The drugs employed were: Tetraethylammonium bromide (TEA) (Boots). The results were essentially the same whether TEA was added to the bath or exchanged for an equimolar amount of Na in the Tyrode. Acetylcholine chloride (Hoffmann-La Roche), potassium chloride (Baker), eserine (physostigmine salicylas), neostigmine (prostigmine methyl-sulphate "Roche") and d-tubocurarine (Nyegaard and Co).

Results.

A. Action of TEA on Contractions of the Rat Diaphragm.

The effect of TEA on the responses to direct and indirect stimulation with various frequencies has been studied. Indirect stimulation: It was found that a concentration of 1 or 2 mM TEA had no significant effect on the responses to either single nerve

Fig. 1. Effects of TEA on neuromuscular transmission in the phrenic nerve diaphragm preparation from rat. Single supramaximal stimulation of the nerve (6 per min). T, tetanic stimulation of the nerve (100 per sec) for 5 sec. Arrow 1, addition of TEA to a concentration of 2 mM. Arrow 2, addition of TEA to a concentration of 3 mM. Arrow 3, addition of TEA to a concentration of 25 mM. D, direct stimulation of muscle. Time mark, 1 min. Rapid kymograph (1.5 mm per sec) during tetanic stimulation.

shocks (6 per min) or tetanic nerve stimulation (100 per sec). Occasionally a very slight increase in the responses to single nerve shocks occurred.

A concentration of 25 mM TEA abolished the responses to single nerve stimuli (6 per min) in the course of 5 minutes. The muscle still reacted to direct stimulation showing that a neuromuscular block was present. The responses to high frequency nerve stimulation (100 per sec) were inhibited by a concentration of 3 mM TEA in the bath. As mentioned above, concentrations lower than this had no neuromuscular blocking action.

These effects are demonstrated in fig. 1.

Slightly more TEA was needed to produce neuromuscular block at 22° C than at 37° C. The increase in response to nerve and muscle stimulation produced with TEA was slightly more pronounced at 22° C than at 37° C.

The neuromuscular block produced with TEA could not be antagonized with neostigmine or eserine. Potassium chloride (50 mg per 100 ml) antagonized the block to some extent. TEA added to the bath containing the nerve alone had no effect on the muscle responses in concentrations up to 50 mM. Higher concentrations affected the nerve so that the muscle responses became depressed. Direct stimulation: After adding d-tubocurarine to a concentration of 2×10^{-5}M in the bath the contractions to nerve

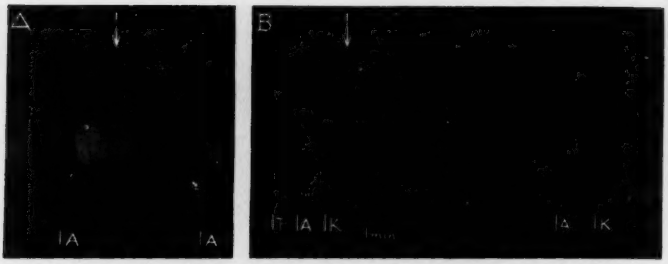

Fig. 2. Effect of TEA on sensitivity to acetylcholine and potassium. Single supramaximal stimulation of the nerve (30 per min). Record A: I_A, rapid injection of 5 micrograms acetylcholine. Arrow, addition of TEA to a concentration of 2 mM TEA. Record B: I_T, rapid injection of 0.2 ml Tyrode solution. I_A, rapid injection of 10 micrograms acetylcholine. I_K, rapid injection of 1 mg potassium. Arrow, addition of TEA to a concentration of 10 mM. Stimulation stopped during injections. Time mark, 1 min.

stimulation rapidly disappeared. The muscle still reacted to direct stimulation.

The excitability was tested by applying gradually increasing current strengths until a just noticeable movement of the lever occurred. This was recorded with minimal friction on the drum before and after the addition of TEA to concentrations of 1 and 2 mM. The pulse length was 1 msec. No change in excitability was observed with these concentrations of TEA. With 10 times higher doses a decrease in excitability occurred.

The contractility of the muscle was tested by recording the maximal contractions of the muscle to direct stimulation. Striated muscle reacts to direct stimulation with maximal contractions provided sufficiently strong stimuli with a duration between 0.6 and 3 msec are used (GJONE 1955). By gradually increasing the pulse durations maximal contractions were reached at 0.6 msec and the contraction height remained at a plateau until 4 msec. This procedure was repeated after the addition of TEA to concentrations of 1 and 2 mM. No change in the height of the maximal contractions occurred. With 10 times higher concentrations some increase in the contractility was occasionally observed. The directly released contractions were not depressed until TEA was added to a concentration above 50 mM.

B. Change in Sensitivity to Acetylcholine and Potassium.

No significant change in the sensitivity to rapidly injected acetylcholine could be detected with a concentration of 2 mM

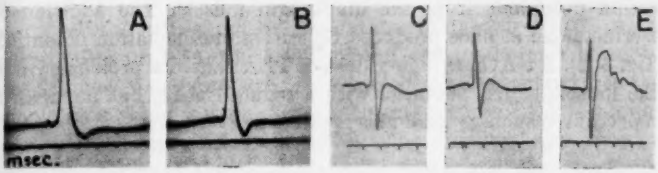

Fig. 3. Effect of TEA on action potentials from phrenic nerve and from diaphragm muscle of rat. Effect of eserine alone on muscle action potentials. Single supramaximal nerve stimulation (30 per min). Pulse length 0.1 msec. Record A: Action potential from untreated nerve. Record B: Action potential 3 min after adding TEA to a concentration of 1 mM. Record C: Muscle action potential from untreated preparation. Record D: Muscle action potential 5 min after adding TEA to a concentration of 1 mM. Record E: After washing the preparation and adding eserine to a concentration of 1×10^{-6} M. Time mark, 5 msec.

TEA in the bath (fig. 2 A). The same was found with potassium. A higher dose of TEA caused a decrease in the sensitivity to acetylcholine, but the stimulant effect of potassium was largely unchanged. In fig. 2 B it is seen that 10 mM TEA in the bath, after a slight increase in contraction height, caused a partial neuromuscular block. This reduced the sensitivity of acetylcholine to zero, but injection of potassium still elicited a muscle twitch. Rapid injections of various amounts of TEA caused no more muscle twitches than equal amounts of Tyrode.

C. Effects of TEA on Action Potentials from the Phrenic Nerve of the Rat.

The phrenic nerve was stimulated with single shocks at 2 second intervals and the action potentials from the same nerve were recorded before and 3 to 5 min after the addition of TEA. In figs 3 A and 3 B it is seen that a concentration of 1 mM TEA had no influence on the action potentials in the phrenic nerve. With 10 times higher concentrations the action potentials were decreased in amplitude, but no repetitive responses were seen.

D. Effects of TEA on the Muscle Action Potentials from the Rat Diaphragm Muscle.

The phrenic nerve was stimulated with single shocks at 2 second intervals and the action potentials recorded from the diaphragm muscle before and after the addition of TEA. In figs 3 C and 3 D it is seen that 1 mM TEA caused no significant change in the muscle

action potentials from the diaphragm muscle. The addition of eserine alone at once produced repetitive muscle action potentials (fig. 3 E). A ten times higher dose of TEA caused a prolongation of the muscle action potential with a reduced peak, but no signs of repetitive responses were observed.

Discussion.

The results of this investigation show that TEA in a concentration up to 2 mM has no neuromuscular blocking action at all on the rat diaphragm. With larger doses a neuromusclar block occurs. This block is similar to that produced by d-tubocurarine in that it can be antagonized by potassium. It differs from the d-tubocurarine block in that it is unaffected by anticholinesterases. In this respect TEA is similar to drugs causing neuromuscular block by a depolarizing action. TEA itself, however, has no depolarizing action and will not stimulate the muscle on rapid intravascular injection, as found in the above experiments. This agrees with the findings of other authors. It is well known that the ability of quaternary ammonium compounds to excite striated muscle is completely lost when 3 ethyl groups are exchanged for methyl on the central N atom (review by RIKER 1953).

Repetitive responses to single nerve shocks occur in frog nerves treated with TEA (COWAN and WALTER 1937). The action potentials of the phrenic nerves from rats, however, were unchanged with lower concentrations of TEA, and repetitive responses could not be observed even with very high doses. This is also supported by the experiment showing the absence of effect of TEA when added to the bath containing the nerve alone with the stimulating electrodes. Repetitive action potentials might, however, still be produced in the terminal portions of the motor axons. When TEA is applied to the various parts of crab nerves, the terminal portions show such phenomena more readily than the main axon (BURKE, KATZ and MACHNE 1953). This cannot be the case with phrenic nerves from rats, since the muscle action potentials after single nerve shocks were unchanged with lower concentrations of TEA. Eserine alone, however, at once produced repetitive responses. The excitability of the muscle membrane to chemical stimulation with acetylcholine and potassium was largely unchanged with a concentration of 1 or 2 mM TEA in the bath. It has been shown that acetylcholine excites only the motor end

plate (KUFFLER 1943), while potassium exerts its stimulant action on the whole muscle membrane (KUFFLER 1945). With higher doses of TEA the sensitivity of the motor end plate to acetylcholine was depressed, while the chemical sensitivity of the muscle membrane was unaffected. In this respect TEA is similar to d-tubocurarine (KUFFLER 1943). The findings also show a similarity between the neuromuscular block and the ganglionic block produced by TEA, because at the ganglia TEA blocks the stimulating effect of acetylcholine but leaves the effect of potassium unchanged (ACHESON and PEREIRA 1946 b).

In doses of 1 or 2 mM TEA seems to have no effect either on any part of the muscle membrane including the motor end plate or on the nerve or its terminal branches in the rat diaphragm. This is of significance as KENSLER (1949, 1950) and SULLIVAN and KENSLER (1950) reported that lower concentrations of TEA had an optimal effect on certain specific neuromuscular blocks in mammals. This action of TEA on the various forms of neuromuscular depression will be the subject of subsequent papers.

Summary.

1) The actions of tetraethylammonium (TEA) on the neuromuscular junction, the phrenic nerve and the diaphragm muscle have been studied separately in isolated phrenic nerve diaphragm preparations from rats. The object of this was to form a basis for the further study of the action of TEA on various forms of neuromuscular block.

2) Concentrations of TEA up to 2 mM caused no neuromuscular block to either single or tetanic nerve stimulation. Higher concentrations caused a neuromuscular block which could be antagonized with potassium but not with anticholinesterases.

3) TEA had no effect on the action potentials of the phrenic nerve in concentrations of 1 or 2 mM. Higher doses caused a lowering in amplitude of the action potentials, but repetitive responses to single nerve shocks were not observed.

4) Muscle action potentials in response to single nerve shocks were unchanged with concentrations of 1 or 2 mM TEA. Higher doses caused a prolongation of the action potentials with reduced peaks. No signs of repetitive responses were observed.

5) Both excitability and contractility of the muscle to direct stimulation were unchanged with concentrations of 2 mM TEA in

the bath. Higher doses usually depressed both the excitability and the contractility.

6) The stimulant effect of rapidly injected acetylcholine and potassium was not affected by a concentration of 2 mM TEA in the bath. Higher doses caused a decrease in the sensitivity to acetylcholine but the stimulant effect of potassium was unchanged.

Acknowledgement.

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The Effect of Low Calcium and of Tetraethylammonium (TEA) on the Rat Diaphragm.

By

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Locke observed as early as 1894 that lack of calcium in the extracellular fluid resulted in a neuromuscular block. Since then Dale and coworkers (1936) have established that neuromuscular transmission is effected by a release of acetylcholine from the presynaptic nerve terminals acting on the underlying specialized part of the muscle membrane, This release of acetylcholine from the presynaptic nerve endings appears to be dependent on the presence of calcium ions, as shown by Castillo and Stark (1952) and by Castillo and Engbaek (1954).

In two short communications, Kensler (1949) and Sullivan and Kensler (1950) reported that tetraethylammonium (TEA) had a marked restoring effect on the neuromuscular block produced by lack of calcium in both the rat and kitten diaphragm preparations. To the author's knowledge, no further reports on this action of TEA have since appeared in the literature.

In a previous paper (Stovner 1957), the effect of TEA on the normal rat diaphragm preparation was investigated. In the present work, the reactions of the isolated phrenic nerve diaphragm preparation in a calcium-deficient medium have been studied, together with the effect of TEA on such a preparation. The object has been to localize and analyze in more detail the restoring effect which TEA is reported to have (Kensler 1949) on this preparation in a calcium-deficient medium.

Methods.

a) Contractions of Phrenic Nerve Diaphragm Preparations.

The isolated phrenic nerve diaphragm preparation from the rat (BÜLBRING 1946) was used with the modifications described in a previous paper (STOVNER 1957). In addition to the Tyrode solution described in that paper three modifications of the bath fluid have been used:

1) The same composition as before but with varying amounts of $CaCl_2$.

2) The same composition but without KCl and with varying amounts of $CaCl_2$.

3) The same composition but substituting the NaCl by 6.5 g sucrose to maintain the solution isotonic in the absence of NaCl.

b) Close Intravascular Injection of Acetylcholine and Potassium on the Rat Diaphragm.

The method of BURGEN, DICKENS and ZATMAN (1949) was used with the modifications described previously (STOVNER 1957). In these experiments the acetylcholine and KCl for injection were dissolved in calcium-free Tyrode.

c) End Plate Potentials from the Rat Diaphragm.

The rat diaphragm with part of the phrenic nerve was fixed horizontally and the electrodes arranged as described in the previous paper. The phrenic nerve was stimulated with single supramaximal shocks and the potentials recorded from a cathode ray oscilloscope. Both the stimulating and recording electrodes were immersed in Tyrode solutions of the above compositions. When neuromuscular transmission was depressed, the end plate potentials were localized by moving the recording electrode. The low voltage monophasic non-propagated potential confined to a strip midway between the costal margin and central tendon was regarded as the end plate potential.

Drugs employed: Tetraethylammonium bromide (Boots), beta-hydroxyethyl-triethylammonium chloride (synthesized at the Chemical Institute, University of Oslo. The substance was obtained in crystalline form and conforming to standards), acetylcholine chloride (Hoffman-La Roche), choline chloride (Hoffman-La Roche), succinylcholine chloride (Burroughs Welcome and Co).

Results.

A. Action of Low Calcium on Contractions of the Rat Diaphragm.

In calcium-free Tyrode solution the responses to single nerve shocks (6 per min) were abolished in 5 to 10 min at 37° C. The preparation still reacted to tetanic nerve stimulation (50 per sec) showing facilitation through the block. After another 15 to 20 min these tetanic responses also disappeared. When the muscle

had ceased to respond to both single and high frequency nerve stimulation, it still reacted to direct stimulation showing that a neuromuscular block was present. The threshold concentration of calcium necessary to maintain minimal muscle responses to nerve stimulation was found to be about 4×10^{-4}M $CaCl_2$. In fig. 1A and B where the temperature was 37° C the bath fluid was changed to one containing 2×10^{-4}M $CaCl_2$. It is seen that the responses to single nerve shocks disappeared, but the muscle still responded to tetanic nerve stimulation (fig. 1A).

At 22° C the failure of the preparation in calcium-free Tyrode bath developed much more slowly. It took from 20 to 30 min for the responses to single nerve shocks to disappear, and the responses to tetanic nerve stimulation diminished gradually over a period of several hours (fig. 2). The concentration of calcium necessary to maintain minimal activity was about 4 times lower than at 37° C. Therefore, when the preparation had been brought to the threshold of failure by lowering the calcium at 37° C, lowering the temperature to 22° C restored function. Lowering the temperature in the bath containing the nerve alone had no effect. This is demonstrated in fig. 3. As it has been shown that potassium has a stronger anticurare effect at lower temperatures, these experiments were also performed in a potassium-free Tyrode solution. The results were essentially similar.

B. Restoring Effect of TEA.

At 37° C TEA restored function completely in a calcium-deficient solution where the calcium concentration had been adjusted to near that required for minimal activity. This effect was not due to an action on the nerve, as addition of TEA to the nerve bath had no effect (fig. 1B). Also the depression of the responses to high frequency stimulation was restored with TEA (fig. 1A). At 37° C TEA did not substitute for calcium in a completely calcium-free Tyrode solution.

At 22° C the neuromuscular transmission could be restored with TEA even when calcium was completely absent from the suspending medium (fig. 2A and B). In fig. 2A the responses to single nerve stimuli had been absent for 2 hours in calcium-free Tyrode solution, the nerve being stimulated all the time with single shocks (6 per min). Addition of TEA at once restored the responses to almost normal contraction height. Often the responses to single nerve stimuli became higher after adding TEA than they were before the calcium-free Tyrode was introduced. The

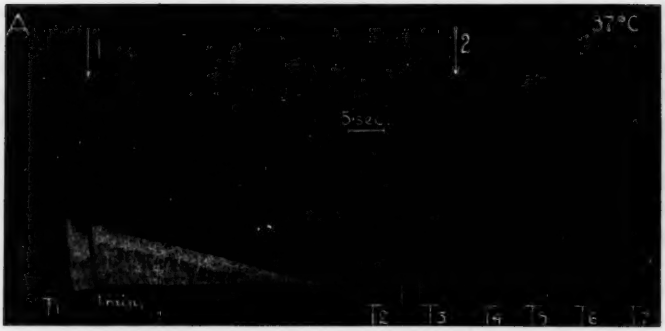

Fig. 1. Effect of lack of calcium on responses to single and tetanic nerve stimuli at 37° C. Restoring effect of TEA. Record A: Arrow 1, change to calcium-deficient Tyrode (2×10^{-4}M). Arrow 2, addition of TEA (1×10^{-3}M). T, tetanic nerve stimulation (50 per sec) for 5 sec. Single supramaximal nerve stimulation (10 per min) between T_1 and T_2. 3 min intervals between the other tetanic responses. Rapid kymograph (1.5 mm per sec) during tetanic stimulation. Record B: Single supramaximal nerve stimulation (6 per min). Arrow 1, change to calcium-deficient Tyrode (2×10^{-4}M). Arrow 2, addition of TEA (1×10^{-3}M) to the bath containing the nerve alone. Arrow 3, addition of TEA (1×10^{-3}M) to the main bath. Time mark, 1 min.

effect of TEA was not temporary but lasted for more than one hour (fig. 2B). However, after several hours of nerve stimulation in a Tyrode solution containing 1 mM TEA instead of the normal 1.9 mM $CaCl_2$, the height of the contractions was reduced. Replacing the normal calcium content of the bath would still restore the contractions. This shows that at this lower temperature as well TEA cannot completely substitute for calcium during prolonged action.

The optimal concentration of TEA for producing "calcium replacement" was found to be about 1 mM. This optimal concentration was the same at 37° C as at 22° C.

The restoring effect of TEA was not dependent on potassium in the solution, as the same results were obtained with potassium-free Tyrode in the bath.

Fig. 2. Effect of lack of calcium on responses to single and tetanic nerve stimuli at 22° C. Restoring effect of TEA. Record A: Arrow 1, change to calcium-free Tyrode at 12^{00}. Arrow 2, addition of TEA (1×10^{-3}M) at 14^{30}. T, tetanic stimulation of the nerve (50 per sec) for 5 sec. 30 min between T_1 and T_2, 2 hours between T_2 and T_3, 6 min between T_3 and T_4. Single supramaximal nerve stimulation (6 per min) throughout the experiment. Rapid kymograph (1.5 mm per sec) during tetanic stimulation. Record B: Single supramaximal nerve stimulation (8 per min). Arrow 1, change to calcium-free Tyrode. Arrow 2, addition of TEA (1×10^{-3}M). Contractions recorded one hour later.

C. Effect of Related Compounds

The introduction of a hydroxyl group in TEA to form beta-hydroxyethyl-triethylammonium resulted in a complete loss of "calcium replacement" value. Choline had a very slight temporary effect, succinylcholine had none.

D. Effect of TEA on Low Sodium Concentration.

Apart from calcium, sodium is the only cation in the Tyrode solution indispensable for the function of the rat diaphragm preparation. Washing the preparations in sodium-free Tyrode made isotonic with 6.5 g sucrose rapidly led to inexcitability. By adding 100 mg NaCl to the 100 ml bath minimal activity was restored. TEA had no effect on this activity.

E. Change in Sensitivity to Acetylcholine and Potassium.

In calcium-deficient Tyrode the stimulant effects of both acetylcholine and potassium were greatly increased (fig. 4A and B). The stimulant effect of potassium was increased more than that of acetylcholine. When TEA was added to a concentration of 1 mM in the bath, the transmission was restored. The sensitivity to acetylcholine was now lowered to about the same as before (fig. 4A). The increased sensitivity to potassium, however, remained after

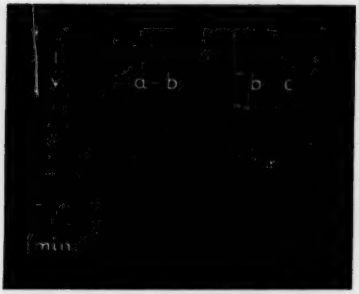

Fig. 3. Effect of temperature on neuromuscular block due to lack of calcium. Single supramaximal nerve stimulation (6 per min). Arrow, change to calcium-deficient Tyrode (2×10^{-4}M). Between a and b the temperature of the bath containing the nerve alone was lowered from 37° C to 22° C. Between b and c the temperature of the main bath was lowered from 37° C to 22° C. Time mark, 1 min.

transmission had been restored with TEA (fig. 4B). If the calcium content of the Tyrode was replaced, the sensitivity to potassium fell within a few minutes. These experiments were performed at 37° C.

F. End Plate Potentials.

End plate potentials were recorded from the rat diaphragm in calcium-free Tyrode at 22° C. This temperature was chosen because TEA antagonized the neuromuscular block due to a complete lack of calcium at this temperature. The effect on the end plate response to graded doses of TEA could then be studied more readily. The end plate region was first localized after a neuromuscular block was produced with d-tubocurarine or magnesium. After washing the preparation thoroughly with normal Tyrode, the calcium-free solution was introduced. In fig. 5 it is seen that the muscle action potentials in calcium-free solution were reduced to a pure end plate response in about 50 min. With further lapse of time the end plate potentials became still more reduced. On addition of a small amount of TEA (1×10^{-4}M in the bath) a marked increase in the amplitude of the end plate potential occurred, without significant change in time course. When more TEA was added a further increase occurred before muscle spikes reappeared. At a certain threshold height of the end plate potential, muscle spikes were initiated. This threshold height seemed to be the same as it was when neuromuscular transmission failed before the addition of TEA. This is seen by comparing figs. 3C

Fig. 4. Sensitivity to the stimulant action of acetylcholine and potassium during lack of calcium at 37° C. Effect of TEA. Record A and B: Arrow 1, change to calcium-deficient bath solution (2×10^{-4}M). Arrow 2, addition of TEA (1×10^{-3}M). I_T, injection of 0.2 ml Tyrode solution. I_A, injection of 5 micrograms acetylcholine. I_K, injection of 0.5 mg KCl. Single supramaximal nerve stimulation (6 per min). Stimulation stopped during injections. Time mark, 1 min.

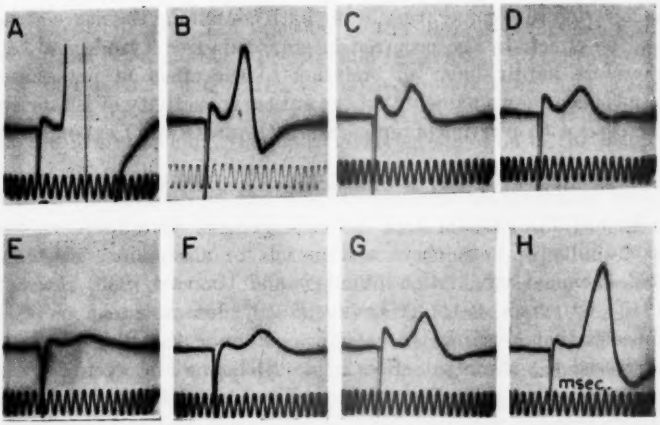

Fig. 5. Effect of TEA on end plate potentials from rat diaphragm in calcium-free Tyrode at 22° C. Single supramaximal nerve stimulation (30 per min). The end plate region was first localized after addition of d-tubocurarine, which was thoroughly washed out. Preparation then placed in calcium-free bath solution. 40 min between recordings A and B. 10 min between the following recordings. Between E and F addition of TEA to a concentration of 0.1 mM in the bath. Between F and G and G and H addition of the same amount of TEA.

and 3G, which both represent end plate potentials just failing to initiate muscle spikes.

Discussion.

1. Effects of Low Calcium.

The present investigation demonstrates that lack of calcium in the suspending medium results in a neuromuscular block of the rat diaphragm preparation. It has also been shown that the block can be overcome temporarily by high frequency nerve stimulation. This is consistent with earlier findings in mammals of BROWN and HARVEY (1940). In this respect the neuromuscular block is similar to that produced by an excess of magnesium (NAESS 1952). However, it differs sharply from that produced by d-tubocurarine.

The finding that a lower concentration of calcium is needed for maintaining minimal activity at a lower temperature, has to the author's knowledge, not been reported previously. Studying mammalian nerves, LAGET and LUNDBERG (1949) observed that the effect of calcium was dependent on temperature. The above described effect, however, was not due to an action on the nerve as lowering the temperature in the bath containing the nerve alone had no effect. It also occurred in potassium-free Tyrode and can therefore hardly have any relation to the effect of potassium. It has been shown namely that the anticurare activity of potassium increases with lowering of temperature (QUILLIAM and TAYLOR 1947).

2. Sensitivity to Acetylcholine and Potassium.

Removal of calcium from the suspending medium increases the excitability of both nerve and muscle to mechanical, electrical and chemical stimulation (ADRIAN and GELFAN 1933, HARVEY 1940, KUFFLER 1944). HARVEY (1940) demonstrated on frog muscles that lowering the calcium content of the Ringer greatly increased the stimulant effect of acetylcholine and even more of potassium. The experiments above (fig. 4A and B) show that this is also true in a mammalian nerve-muscle preparation in a calcium-deficient medium. These findings indicate that the neuromuscular block is presynaptic in origin and not due to a reduced sensitivity of the motor end plate to the transmitter substance. It thus differs markedly from the neuromuscular block caused by d-tubocurarine as this substance blocks both the transmission of nerve impulses and the stimulating action of acetylcholine (KUFFLER

1943). In a previous paper (STOVNER 1957) it was shown that a concentration of 1 mM TEA on a normal preparation had no effect on the stimulant action of acetylcholine and potassium. The present experiments (fig. 4A and B) demonstrate that when TEA is added the enhanced sensitivity to acetylcholine in a calcium-deficient medium is lowered towards normal, while the even more increased sensitivity to potassium remains. It is known that potassium stimulates the whole muscle membrane (KUFFLER 1945), while acetylcholine excites only the motor end plate (KUFFLER 1943). It seems therefore, that TEA normalizes the motor end plate in a calcium-deficient medium, but does not stabilize the rest of the muscle membrane to the same extent as calcium. This may be a question of affinity for or ability to penetrate into the two types of membranes by calcium and TEA ions.

3. Restoring Effect of TEA.

The present investigation shows that TEA in a concentration of 1 mM restores neuromuscular transmission when this has failed as a result of lack of calcium. This concentration of TEA has no observable effect on the function of the various components of the normal rat diaphragm preparation (STOVNER 1957). From the work of CASTILLO and STARK (1952) it appears that the release of acetylcholine from the presynaptic nerve terminals fails in a medium low in calcium. The most likely explanation of the restoring effect of TEA is therefore that it specifically effects this release of acetylcholine and thereby restores transmission. This is supported by the finding that addition of TEA to the bath containing the nerve alone did not restore function. The increase of the end plate potential following addition of TEA is more direct evidence of an increase in the output of the transmitter. As mentioned above, it was found that TEA lowered the increased sensitivity of externally applied acetylcholine towards normal in a medium low in calcium. In spite of this reduction in sensitivity to externally applied acetylcholine, the end plate potential, set up by endogenous release of acetylcholine, is increased. The most likely explanation of this is that considerably larger amounts of the transmitter are released with each nerve impulse when TEA is added. This is exactly what occurs when calcium is added to a preparation where neuromuscular transmission has failed because of lack of calcium (CASTILLO and STARK 1952).

The excitability of the normal muscle membrane is unchanged in the presence of a concentration of 1 mM TEA (STOVNER 1957). The present experiments with end plate potentials (fig. 5) demonstrate that in calcium-deficient medium as well TEA does not change the excitability of the muscle membrane to any significant extent. This is seen from the fact that the same threshold of the end plate potential is needed to initiate a muscle spike after the addition of TEA as before (fig. 5C and G).

ECCLES and MAC FARLANE (1949) have shown that inhibition of the end plate cholinesterase causes a pronounced increase in the time course of the end plate potential. In fig. 5 it is seen that TEA produces predominantly an increase in amplitude of the end plate response. This is in good agreement with the findings of BARLOW and ING (1948) and KENSLER and ELSNER (1951) that TEA does not inhibit the cholinesterases from either humans or rats to any significant extent.

4. The Specificity of the Action of TEA.

It was shown that when the function of the rat diaphragm preparation had failed due to a depletion of sodium ions, these could not be replaced to any extent by TEA. LORENTE DE NÓ (1948, 1949) reported that he was able to restore function depressed by sodium depletion with TEA in types B and C nerve fibres from frog. With A fibres, however, he obtained no effect. Furthermore, LORENTE DE NÓ found that this effect was also shared by a number of related quaternary ammonium compounds, and also by the beta-hydroxy derivative of TEA. In the above experiments, however, it was shown that the introduction of an OH group in the beta position in TEA resulted in complete loss of calcium replacement value. The ability of TEA to replace calcium at the mammalian neuromuscular junction seems therefore to be a more specific effect of TEA than its sodium replacing ability in frog B and C nerve fibres.

5. The Relationship Between TEA Ions and Calcium Ions.

The question of how a quaternary ammonium compound like TEA can replace calcium ions at the mammalian neuromuscular junction is interesting. COWAN and WALTER (1937), studying the antagonism between large doses of TEA and calcium on frog nerves, suggested that these ions competed for strategic positions along the nerve membrane. They pointed out that the calculated radius in solution of TEA ions and calcium ions was very similar.

At the myoneural junction, Ing (1936) has drawn attention to a striking correlation between the ionic radius of simple quaternary ammonium compounds and their neuromuscular blocking action. Similarity in ionic size is therefore a possible deciding factor.

It is possible that, also in the presence of a normal amount of calcium in the surrounding medium TEA may cause an increased output of the transmitter. Such an effect will not be observable in a normal preparation, because the end plate potential is already supramaximal. With an intact end plate cholinesterase, the muscle responses to nerve stimulation will therefore be unaffected. In a curarized preparation, on the other hand, the end plate potential is below the threshold for initiating muscle spikes. By studying the anticurare activity of TEA in detail it should be possible to obtain an answer to this question. This will be the subject of a subsequent paper.

Summary.

1) The effect of lowering the calcium content on the neuromuscular transmission of the isolated phrenic nerve diaphragm preparation of the rat has been investigated at 37° C and at 22° C. The threshold concentration of calcium necessary to maintain minimal activity was found to be about 4 times lower at 22° C than at 37° C. This was not due to an action on the nerve, and was independent of the presence of potassium ions.

2) A concentration of 1 mM tetraethylammonium (TEA) completely restored the neuromuscular transmission depressed by a deficiency of calcium, provided the calcium content had been adjusted to that necessary for minimal activity at 37° C. At 22° C TEA restored transmission for more than an hour in a completely calcium-free medium. During prolonged action, however, TEA could not replace calcium completely at this lower temperature either. This effect was also independent of the presence of potassium. Neither the beta-hydroxy derivative of TEA nor choline had a restoring effect.

3) When the function of the rat diaphragm preparation was depressed because of sodium depletion, TEA had no restoring effect.

4) The end plate potential obtained in a calcium-free Tyrode solution was increased in amplitude without change in time course, when TEA was added.

5) In a calcium-deficient solution, the muscle contractions produced by rapid intravascular injection of acetylcholine and potassium were greatly enhanced. When neuromuscular transmission was restored with TEA, the sensitivity to acetylcholine returned to normal, but the enhanced sensitivity to potassium remained.

It is suggested that TEA effects the release of acetylcholine from the presynaptic nerve endings like calcium, but does not seem to stabilize the muscle membrane to the same extent as calcium.

Acknowledgement.

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